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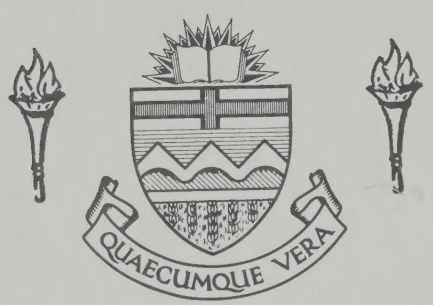

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April 26, 1991

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled TRACHEOBRONCHIAL MUCUS RHEOLOGY IN LUNG CANCER submitted by Dr. JOSE GUSTAVO ZAYAS ZAMORA in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Experimental Medicine, Department of Medicine - Pulmonary Division.

[redacted]

April 26, 1991

I wish to dedicate this thesis first to my loving family, my wife, Tey, and my children, Julieta, Ana, Paula and Jose Gustavo for their personal support and encouragement; to my parents for their guidance, Rosa Zayas and Juan Zamora; to all my brothers and sisters; also to my dear late friend and colleague, Napo (Dr Napoleon Gutierrez Quintanilla) who was murdered in Usulutan, El Salvador, while searching for social justice; and finally, to the people of El Salvador in support of their struggle for peace.

TRACHEOBRONCHIAL MUCUS RHEOLOGY IN LUNG CANCER

ABSTRACT

Lung cancer is a major worldwide health problem. The link between lung cancer and cigarette smoking is firmly established, but the role of long-term impairment of mucus clearance resulting from smoking has not been well-defined. The basic question of this thesis was therefore: How does impairment of mucus clearance relate to lung cancer?

The thesis begins with a review of the structure and function of normal mucus. The experimental studies involved subjects undergoing diagnostic bronchoscopy, including four major categories: nonsmokers, smokers, exsmokers and subjects with lung cancer. Mucus was collected using a bronchoscopic method. The viscoelastic properties were determined by magnetic microrheometry.

Our first study demonstrated that smoking is associated with a long-lasting, progressive alteration in the viscoelastic properties of mucus. In all smokers, with or without cancer, the mucus was predicted to be less easily clearable by coughing. Mucus from cancer patients was inappropriate for both ciliary clearance and cough clearance. These changes were not age-dependent.

Based on these findings we hypothesized that rheological abnormalities would be seen in the mucus of the lung with cancer compared to the one without cancer. Hence, in the second study, paired samples were obtained from both mainstem bronchi. Subjects were grouped according to the presence or absence of a radiological abnormality. The rheological properties of mucus, (particularly tan δ_{100}, a parameter inversely related to elastic recoil) strongly suggested which was the abnormal side. Mucus from the side with the radiological lesion had a lower value of tan δ_{100} ($p = 0.004$, paired t test). All eight cancer patients fit a common mucus rheological pattern (lower tan δ_{100} on the affected side, $p = 0.007$). Using both the rigidity factor and the recoil factor, and assisted by other diagnostic tools, our accuracy in distinguishing the cancerous lung from the healthy one is enhanced. We have designed a triad of rheological data that enables us now to clearly separate subjects at high risk of developing lung cancer from those at lower risk. We have thus achieved the main objective of the thesis: to develop a technique to be used as a diagnostic tool in the struggle against lung cancer.

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I am hopeful that the research presented will not only advance the cause of mucological research, but also present new directions, new concepts and new techniques to aid in our ultimate goal of combating and eradicating lung cancer.

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Chapter I

INTRODUCTION

Lung cancer continues to be a worldwide major health problem. Presently it is the most common tumor in the world (1) and the leading cause of cancer mortality (2, 3). The rate of lung cancer remains steadily high in men and is on the increase among women (4), similar to the rate observed in chronic obstructive pulmonary disease (COPD) (5, 6). Although 80 to 90 percent of lung cancers are preventable (4, 7, 8) and despite all of the efforts and resources dedicated to fight lung cancer, the mortality rate still is very high. Primary prevention, not to smoke or smoking cessation programs, or secondary prevention such as developing techniques or methods that could identify or predict subjects who are in imminent danger of developing lung cancer, appears to be the only hope in winning the real battle against this very deadly form of cancer.

Tobacco consumption is related to cancer in the bladder, the cervix, oral cavity, larynx, esophagus and stomach in addition to lung cancer (4, 8). Tobacco smoking is also clearly associated with chronic bronchitis, emphysema, and COPD (9, 10, 11). Cigarette smoking is the greatest single causal factor in the risk of developing lung cancer. National campaigns against smoking have been very successful in the developed countries (12, 13). Regardless of the enormous amount of information provided by these campaigns, which show the link between cigarette smoking and lung cancer, it is highly disturbing to discover that more and more young people are starting to smoke cigarettes (14).

It is also highly distressing that tobacco manufacturing companies are shifting their target market to the underdeveloped countries. Tobacco consumption rates in these countries is continuously increasing (4, 15). This will have a devastating effect on the highly fragile economies of these countries. After a "normal latency period" of approximately 40 years, one can easily predict that the number of lung cancer cases will start to appear in a steadily increasing form. As a consequence, an increasingly larger proportion of the national budgets will have to be used to ameliorate the suffering of the victims of

smoking. Thus, one can even foresee a spectrum of social unrest increasing as people demand more attention than the government can provide due to lack of resources.

Different prospective and large scale screening studies have been carried out attempting to find a method of early detection of lung cancer (16, 17, 18, 19). In these studies, several combinations of data, e.g. clinical, radiological, biochemical and pathological, have been used. Parameters considered in various studies include race, genetic predisposition, diet, and type of work. Often various prestigious centres were simultaneously involved.

Recent attention has been focused on airway obstruction. This approach may be based on Skillrud's observation that subjects with COPD have a higher risk of developing lung cancer ($p=0.024$) (18). A number of studies also have shown that chronic smoking leads to abnormalities in mucus clearance in humans (20), which may develop as early as one year after the smoke exposure was initiated. Clearance of airway mucus requires an efficient interaction of mucus and cilia. Alterations in the mucociliary apparatus are related to pathophysiological changes in the bronchi and bronchioles, leading to chronic bronchitis and chronic bronchiolitis. Accumulation of mucus and histological changes of the epithelium are characteristic of alterations in both the mucociliary apparatus and these two diseases. Airway obstruction is believed to result when mucus accumulation and changes in the epithelium occur.

The anatomical changes that take place in the airways with cigarette smoke exposure are well documented in humans and in animal models (21, 22, 23, 24): 1) there is denudation of the ciliated epithelium; 2) the number of goblet cells is increased; 3) hypertrophy of the submucosal glands occurs along with certain degrees of metaplasia; and 4) an increased quantity of mucus is also documented. These changes are observed not only in subjects with chronic bronchitis but also in "healthy" smokers.

Studies of the genetic events related to the development of lung cancer have rapidly advanced in the last decade (25). Cell proliferation and differentiation in the airways have to be balanced. The presence of

pluripotential cells in the airways, like the basal cell and the secretory cells – goblet and Clara cells, makes this process more complex. The development of airway neoplasia is a multistep process that involves a number of genetic alterations (26, 27, 28). Minna (25) has documented evidence of damage in recessive and dominant genes that occurs during the development of lung cancer. DNA adducts, chromosomal deletions, particularly a deletion that occurs in the region 3p, and abnormalities in mRNA expressions, are examples of the damage reported. Some of these are considered to be earlier steps in the neoplasia pathogenesis. Of relevance to the topic of mucus in lung cancer is the contention that glycoprotein secretion is under genetic control, and could therefore be modified with the appropriate genetic alteration (29).

Most of the current research on the respiratory tract is not directed at the prevention of lung cancer (4), but is curative-oriented or palliative. Knowing that lung cancer can be prevented, encouraging smoking cessation in smokers is a better approach. Changing attitudes about taking up cigarette smoking, particularly among young people, may be the best approach of all to cancer prevention. The World Health Organization (WHO) has sponsored several congresses to address the multifaceted problems of lung cancer.

Smokers, even light smokers, have already started this multistep process. Detecting and identifying those at risk of developing lung cancer may provide those so identified with their best alternative. Our contribution, if any, to this worldwide concern is only at the secondary prevention level. We have been analyzing, specifically in the airways, the role that mucus may play in this neoplastic process, and its potential role as a diagnostic tool.

In the second chapter of this study we will present a literature review about normal mucus. The scope of this review is very large, hence some topics are not covered in depth. The major aim of the review is to look for existing commonalities of mucus among the different systems of the body. Some similarities we have observed are that mucus functions to maintain a steady local homeostasis, mucus has a common basic physical structure, mucus is produced in the same types of cells, and within those cells the macromolecules are assembled in an identical microsystem of organelles. An issue of current

debate in mucus research is whether the mucus macromolecules assembly is under genetic control.

Another aim of the review is to try to gain a better understanding of the normal physiological function of mucus. This will help us in the future to interpret appropriately any alterations resulting from internal or external factors.

Mucus is produced in minimal quantities in normal airways. The difficulty of collecting mucus from normal subjects has been recognized as one of the greatest obstacles to the study of mucus (30). Dr. Malcolm King and his coworkers developed a bronchoscopic technique to collect tracheal mucus from normal subjects (31). Dr. King also modified the magnetic rheometer to allow researchers to analyze microquantities of mucus (32). With these pioneering steps, Dr. King and colleagues have been able to define the normal baseline for the rheological values of the tracheal mucus in dogs and in humans, and thus set the stage for the studies described here.

Hence, we are studying the physical properties of the respiratory tract mucus in humans and in animals using an advanced technique – the magnetic microrheometer. Abnormalities in mucociliary clearance, which could relate to both obstruction and cancer, are regarded with high priority in our approach. We are therefore concentrating our efforts on studying all of the alterations that occur in the mucociliary system as a result of external factors, such as cigarette smoke exposure. We will correlate these changes with the development of airway pathologies, specifically lung cancer.

In the third chapter we will present the results obtained in the analysis of tracheal mucus collected from patients undergoing diagnostic bronchoscopy. The aim of this study is to examine whether cigarette smoke exposure alters the rheological properties of mucus in humans as it does in animals. We will assess the effects that this change may exert on the mucociliary clearability system in smokers and exsmokers, as well as in subjects with lung cancer. In addition to rheological data, other clinical and nonclinical diagnostic parameters were included. Based on these alterations, we may support or disregard the hypothesis that abnormalities of mucus rheology and clearability could represent a risk factor in the development of lung cancer.

The fourth chapter is based on the findings of the previous chapter. We present the data obtained from the analysis of mucus collected from the right and the left mainstem bronchi. Because we found rheological differences in the tracheal mucus in patients with and without lung cancer, independently of their smoking history, we are interested in improving the specificity of our rheological method. It may be possible to find rheological differences in mucus from the side with the lesion, compared to the side with no lesion. The fact that anatomophysiological differences exist between the side with the cancer compared with the "normal" side has attracted our interest. Do these differences in mucus properties determine a rheological pattern typical or characteristic of lung cancer? We also want to further explore our finding in a former study that cough clearability is also abnormal in cigarette smoke-exposed subjects. Given these previous considerations, our ultimate purpose is to develop a technique using the rheological analysis of mucus that will help us to detect and identify people who are in risk of developing lung cancer.

Some types of cancer have been brought under control. This control was only made possible when methods of prevention were developed. The Papanicolau test is perhaps the best example of an effective method of detecting people at risk of developing cancer. This single factor led health care workers to achieve real curative measures. As a result, cancer of the cervix is almost rare today. This should be the ultimate aim for other types of cancer, including lung cancer, i.e., to diagnose or identify developing cancerous lesions at a stage when the outcome can be altered.

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Chapter II

COMPARATIVE ASPECTS OF MUCUS STRUCTURE AND FUNCTION

Introduction

The epithelia lining all the major body cavities are in contact with mucus, a common secretory product. Mucus in turn is in contact with the three main presentations of materials: gases in the lung, liquids in the stomach and bladder, and solids in the colon. In these three component systems, mucus is produced by similar cells or tissues: single cells (goblet cells) or organized groupings (submucosal glands); mucous cells and serous cells. Cells producing mucus, and the mucus itself, are exposed to and may respond in a similar fashion to common agents such as tobacco products and other carcinogens, as well as to neurotransmitters, inflammatory mediators, among others.

Mucus fulfills its basic role of *protection* in conjunction with ciliated and nonciliated cells. The protective role of mucus can be observed when it acts as a trapping substance in the airways or as a buffer coating for the gastric mucosa. Mucus *lubricates* some tissues and also *facilitates* the passage of the spermatozoa through the cervical canal (1).

Mucus is the response or result of a physiological need, and both the quality and quantity of mucus can change accordingly. Sometimes this change may occur rapidly and be shortlived, such as in the case of exposure to allergens. As soon as the exposure to the allergen stops, mucus production returns to normal. The changes in mucus can also be cyclical (as in the cervix corresponding to the menstrual cycle) or seasonal (2). In other situations mucus changes may be more prolonged and the process may eventually lead to irreversible alterations in the mucus. This finally may result in altered mucus-cellular interaction. In this review we will emphasize respiratory tract phenomena.

The mucus-cell binary system

Two important components that have been ignored or inadequately studied are mucus and the binary system of the mucus and the cell. Lack of means to collect and analyze the mucus has been considered to be the main obstacle in advancing the study of this material. Mucus is often considered unpleasant to work with and inelegant to talk about both professionally and culturally. Mucus, however, is a very important material.

In past studies, mucus has been considered an inert waste blanket whereas the cilia have been recognized as the most important active component of the clearance system. Mucus-producing cells and the mucus or secretion interact with other cells that may or may not be ciliated. The cilia have been studied in great detail using various techniques and advanced technologies. Cilia, however, are only part of the ciliated cell. Previous studies have also focused on viewing either component, cilia or mucus, as isolated units.

The binary or bicellular system, composed of a mucus-producing cells and either a ciliated cell or nonciliated cells, acts as an anatomophysiological unit. It is important therefore to focus on the mucus and cilia as a binary system to understand their reciprocal roles. We should adopt the latter integral approach when studying this intricate and complex process. Mucociliary interaction is most evident in the airways; however, there is also an interaction with the nonciliated and non-mucus producing cells of the digestive tract. We must closely examine this interaction to understand how this anatomophysiological unit functions.

Changes in either component of this bicellular system can produce a variety of effects. In the airways we can examine the mucus-cilia unit where the local homeostatic process occurs. We should also bear in mind that the same bicellular unit exists in the digestive tract and in the genitourinary tract. We may even expect to find a similar reaction to external and internal factors in these other systems. Common cellular factors could show up because of common exposure to carcinogens. For example, tobacco carcinogens may travel to the gastrointestinal tract after being cleared from the lung via the esophagus, but

also may travel to the bladder or to the cervix by circulatory pathways. In other words, we are proposing that any organ in the body that contains mucus-secreting cells has the capability of demonstrating the cellular changes associated with the carcinogenic process.

Mucus and the different types of cells and tissues that form part of the bicellular system play a role similar to the one played by the blood system. Mucus, like plasma, acts as a vehicle and as a medium in which various physiological processes take place. Mucus has an electrolyte content similar to plasma and bile (3, 4). Mucus is also in contact with several systems of the body. We can make an analogy to a sample of blood taken from any area of the body for analysis. For instance, a sample of mucus from one area of the body could conceivably be used to detect alterations occurring in other areas of the body such as the (assumed) common abnormality in cystic fibrosis secretions. An increment in the number of neutrophils in a blood sample may alert us to the presence of an acute alteration such as an infection in any part of the body and direct us to its source. In another instance, a modification in the chemical composition in the plasma may indicate a metabolic alteration. We are therefore suggesting that alterations in the quality and quantity of mucus might indicate the possibility of pathological changes, and, even further, the possibility of malignancy. These alterations in mucus might occur in common throughout the body, although perhaps to different degrees.

Basbaum (5) outlines the steps in this multistage process demonstrating how the airway epithelium responds to different injurious agents. She proposes that these agents may modulate the biosynthesis of airway secretions via genetic regulation. She suggests that mucin gene transcription induced by injury may represent a primary event in the development of mucus cell metaplasia and hypersecretion.

Mucus secreting cells

The epithelial structure varies in the organs that produce mucus. The epithelia may be stratified or pseudo-stratified. In the airways the epithelial structure is pseudo-stratified and columnar with ciliated cells. In the digestive tract the structure is simple columnar. In the cervix it is cryptical with secretory and ciliated cells, whereas in the endometrium it varies according to the phase of the menstrual cycle. The basal cells and the secretory cells are both considered to be stem cells in the airways (6, 7).

Mucus is produced mainly in two types of secretory cells of the epithelium - mucous cells and serous cells. Secretory cells are spread over the epithelium of the different systems or form glands located in the submucosa. Glands may contain both mucous cells and serous cells. Secretory cell types vary not only in their numbers but also in their characteristics and functions in the respiratory, digestive and genitourinary systems. Secretory cells can be identified by various histochemical characteristics of the glycoproteins, according to the number of granules they contain or by their ultrastructural features.

The cell has three basic features. It is surrounded by a membrane to separate it from or connect it to the surrounding cells or environment. The interior consists of the cytoplasm which in turn contains several organelles. It has a nucleus in which the genetic control is located. The cellular membrane is a tight two-layer structure that separates the interior of the cell from the exterior. The membrane has active and passive passages of elements on both sides. The membrane maintains a critical homeostatic balance within the interior of the cell. Ions and molecules are transferred through the membrane constantly. Within the membrane there are regions known as *gap junctions* which act as attachment and communication channels. The *desmosome* is another type of specialized structure that allows two cells to attach mechanically.

The cell has three faces. The upper face of the cell makes contact with the lumen, the lateral face connects the cell to surrounding cells, and the basal face attaches the cell to the basal lamina (8). Some researchers have found that in the pseudostratified epithelia of the airways not all columnar cells are directly attached to the basal lamina (9).

The *nucleus* of the cell is surrounded by a two layer membrane that contains many specialized pores whose function is still quite unknown. It appears that the nuclear membrane like the cytoplasmic membrane fulfills the functions of protection and exchange. The exchange occurs between the cytoplasm and the RNA and DNA matter inside the nucleus. The proportion and organizational array of the chromosomal DNA inside the nucleus gives us a broad understanding of the specific activity undertaken by that cell. The heterochromatic nucleus represents a nucleus with coiled up DNA, while the euchromatic nucleus has unwound DNA that needs to be transcribed to RNA. The nucleus is the centre of the synthesis of proteins. The nucleolus contains the DNA sequence that codes for ribosomal RNA which represents or suggests the level of protein synthesis of a particular cell. A cell with a high level of protein synthesis could have several nucleoli.

The *cytoplasm* is located between the cellular and nuclear membranes. Within the cytoplasm are the organelles which have special functions and other components such as particles, filaments, etc.

The *ribosome* is comprised of a pair of subunits. Each subunit contains RNA and proteins. The ribosome is intimately related to protein synthesis, a fundamental function of the cell. The RNA messenger containing the coded sequence of the polypeptide's aminoacids is attached to the ribosomes between the two subunits.

Polyribosomes, which are several ribosomes in sequence, are attached to an RNA messenger. Polyribosomes can be found free in the cytoplasm if the polypeptide is to be used in the cytoplasm. If the polypeptide is to be exported, the polyribosomes are connected to the endoplasmic reticulum.

The *endoplasmic reticulum* is a set of compartments of different shapes and functions. Each compartment is surrounded by a membrane. The endoplasmic reticulum is present in three forms: the rough endoplasmic reticulum, the smooth endoplasmic reticulum and the Golgi complex. The rough endoplasmic reticulum (RER) is a set of cisternae of varied extension, narrow in width, with several polyribosomes attached to the cytoplasmic side. Polypeptides meant for exportation are not in contact with the cytoplasm. The

ribosomes which produce the polypeptides are attached to the membrane of the RER to facilitate the entrance of the polypeptides to the cisternae.

The *smooth endoplasmic reticulum* (SER), a network of tubules that contains several enzymes in their membranes, is capable of enzymatic and metabolic processes such as detoxification. The SER is prominent in the liver and has also been found in some cells of the respiratory system such as the type II pneumocytes and the Clara cell.

The *Golgi complex*, another cisternal structure, appears to serve two basic functions. First, it is the exportation protein packaging centre and second, it is the main mucopolysaccharide "factory". Several granules containing hydrolytic enzymes that form the *lysosomes* are located near the Golgi complex. There seems to exist an intimate relationship among the RER, the lysosomes and the Golgi complex (RERLGC), which suggests they may act as a functional unit.

The *residual body* is a cytoplasmic granule that contains nondigestible material or residues for a long period of time. The *mitochondria* are another type of organelle and are the site of the oxidative phosphorylation process.

Several, if not all, of the membrane-bound compartments of the cytoplasmic matrix act as virtual extracellular compartments. Endocytosis and exocytosis are the mechanisms that the cell uses to incorporate or to discharge several products. The most widely accepted view is that mucus-producing cells use exocytosis as a mechanism to discharge mucus. Verdugo (10) has modified this view and suggests that mucus is expelled in a condensed form that hydrates immediately, thereby expanding the molecule.

The composition of mucus

Mucus is a mobile layer. The movement of the mucus lining the epithelium occurs when it is propelled by interaction with the cilia (airways and cervix), the airflow (cough) or the muscle (intestine). The speed of the mucus movement will basically depend on its physical properties. The biochemical composition plays a critical role in determining these physical properties, and should be constant in the absence of external irritants. Ninety-five percent of mucus is

water and the remaining 5% is made up of proteins, carbohydrates, salts, *et cetera*.

The physical properties of the mucus vary in response to the need to maintain homeostasis and to function adequately. The variation in mucus properties also occurs as a result of local or generalized, direct or indirect insult to the epithelium. Hormonal effects can also be seen in the digestive and cervicoendometrial tract.

The physical properties of mucus, in particular its viscoelasticity, depend principally on the type and amount of glycoproteins and probably also on other components such as linking proteins and high molecular weight DNA (11). Mucus clearability, which is determined by its physical and chemical properties, may be altered by several internal and external factors. One external factor of interest to us, because of its devastating effects, is tobacco.

The mucus macromolecule is unique. It has a similar basic biochemical structure in the different systems in which it is present. The mucus macromolecule consists of a protein core made up of approximately 800 amino acids mostly surrounded by short carbohydrate chains. The chains are linked by O-glycosidic bonds to the amino acids threonine and serine. These glycoprotein subunits are interspersed with naked peptide regions susceptible to proteolytic digestion (3). Disulphide bridges, susceptible to sulphydryl bond breaking agents, play an important role in maintaining the structure of the mucus as do hydrogen bonds between the oligosaccharide moieties (12).

Four major types of intracellular glycoproteins can be identified: i) neutral glycoprotein; ii) sialylated glycoprotein with sialic acid sensitive to the enzyme neuraminidase; iii) sialylated glycoprotein resistant to neuraminidase; and iv) sulfated glycoprotein. Whenever a neuraminic acid is substituted, it is known as sialic acid. N-acetylneuraminic acid is the only sialic acid considered important in humans.

The different types of glycoproteins can be identified by using staining techniques such as Alcian Blue (AB) and periodic acid Schiff (PAS) (13). Isolated or gradually progressing alterations of the internal structure of the glycoprotein components may cause deleterious effects that are generally not

noticeable until the changes seriously affect bodily functions. Initial alterations in the glycoprotein components, reflected in the mucus properties alone or in the bicellular interaction, may decode or shed some light on understanding the development of a malignant process like lung cancer or crippling entities such as chronic obstructive pulmonary disease (COPD).

Mucus in the respiratory system

The respiratory system performs one of the most vital functions of the body. The respiratory process consists of an appropriate gas exchange occurring in the lungs between blood and air. It provides oxygen to the organism and removes carbon dioxide. To execute this function, air has to travel through a series of passages, interact with several types of tissues and be in direct contact with mucus, until it reaches the alveolus where the gas exchange takes place. Air must be cleaned, warmed and humidified.

There are several mechanisms to protect such a vital function. Air is in contact with many bacteria, pollutants and particles in the environment. Many of these components are innocent participants, but others are very harmful, particularly the enormous number of natural and industrial pollutants. Two main complementary mechanisms act to maintain the airways as free as possible of foreign bodies. The mechanisms of protection are the bicellular clearance and the cough clearance. The former constitutes the first line of defense and the latter is the backup or reinforcement if the first one is unable to carry out the task. The bicellular clearance is synonymous with mucociliary clearance and represents the interaction of the mucus-producing cell with the ciliated cell. The mucus produced has to have the optimum physical properties to couple with the normal cilia.

In the respiratory system we most often encounter mucous cells and glandular cells, although basal and nonciliated columnar cells are also present. There are two other cells which either produce or potentially may produce mucus or mucus-related material. These are the Clara cell, found mainly in bronchioli with internal diameters of 0.1 - 0.2 mm or less, and the type II

pneumocyte. Clara cells and type II pneumocytes contribute with their secretion to give mucus its unique physical properties. Type II pneumocytes in the alveoli produce surfactant materials that provide a number of functions, including an antiadhesive function which prevents the collapsing of structures like the alveoli and the bronchi. These pneumocytes also prevent excessive retention of fluid in the airspaces or flooding of the alveolus.

Mucus is considered to have two phases: a gel phase and a sol phase. The sol phase can be separated by centrifugation. In the sol phase we find the presence of many of the plasma proteins. Some plasma proteins are still attached to the gel phase and can be removed by other means. The vast majority of bronchial glycoproteins remain in the gel phase after centrifugation. Several methods can be used to solubilize the gel phase: hydrogen-bond-breaking reagents such as urea, disulphide bond-cleaving reagents, proteolytic enzymes and ultrasonic treatment. Several techniques are also used to remove plasma proteins from bronchial glycoproteins, such as ethanol fractionation, ultracentrifugation, and gel filtration.

The sugar composition of bronchial glycoproteins obtained in this way is similar to other mucus glycoproteins. There exists a determined percentage in the composition of the different sugar components of the glycoproteins which is also directly related to the composition of the blood groups. For instance, in blood group A, Roberts (14) found fucose, galactose, glucosamine, galactosamine, and sialic acid well represented but no mannose nor uronic acid. Blood group activity at some point determines the variation of the sugar content of the airway glycoproteins. The relationship between blood group activity and the physical properties of mucus, if any, has not been established.

The monosaccharides that contribute to form mucus glycoproteins all consist of a six-membered pyranose ring. The pyranose ring may have several conformations, but it is the "chair" form that interests us the most because this form provides a better illustration of the stereometric or tridimensional structure of the monosaccharides. If a substitution occurs in this ring, we can expect this substitution to happen in the axial plane or in the equatorial plane (15). Alpha and beta isomers are mirror images of the same group. Galactose may be

considered to have a chair-form pyranose ring that can have substitutions on positions 2, 3, 4, or 6.

When monosaccharides are formed, sugar sequences have a fixed orientation; however, a sugar sequence with a different stereoisometric orientation would potentially form dozens of atypical monosaccharides. Such changes may provide the basis for slowly developing dramatic events. We might expect that such alterations could cause oligosaccharides to take different shapes, and almost certainly the resultant oligosaccharides and ultimately the mucus would have different physical and physiological properties.

An oligosaccharide chain may have a linear structure or a branched structure. Whether linear or branched, they have only one reducing end that contains N-acetyl-galactosamine as the terminal part. If the chain is branched, the other terminals are not reducing ends.

Two and perhaps three types of bronchial glycoproteins have been found by means of subfractionation procedures. Havez (16) and Lamb (17) found neutral glycoprotein, sialylated glycoprotein, and sulfated glycoprotein. The results of Havez are still highly contentious as Roberts (18) and Boat (19) did not find distinct classes of sialylated or sulfated glycoproteins. The composition of acidic and neutral glycoprotein varies quantitatively depending on the disease. Compared with the acidic glycoprotein content of sputum in chronic bronchitis, cystic fibrosis shows an increase in acidic glycoproteins, whereas asthma and lung cancer show a lower content of acidic glycoproteins. O-glycosidic linkages occur between threonine and serine in the polypeptide backbone and N-acetylgalactosamine in the carbohydrate side chains. The oligosaccharide units show a wide heterogeneity regarding acidity and molecular size (18).

The neutral units are small in size and run from single residues to chains with up to ten sugars. The acidic oligosaccharides are longer than the neutral oligosaccharides; the more heavily sulfated chains contain 20 or more sugar residues. An increase in the size of the oligosaccharide generally results in an increase of sulfate; on the contrary, sialic acid content is higher in smaller acidic oligosaccharides (14). Regarding acidic oligosaccharides, it has been

suggested that sialic acid and sulfate occur on the same oligosaccharide. No firm evidence has been found yet for two distinct classes of acidic oligosaccharides. There is still a possibility that some oligosaccharides may contain sialic acid and other oligosaccharides may contain sulfate groups.

Up to half of the total of the amino acids in mucus are made up of serine, threonine and proline (14). Mucus proteins can be contaminated with or mixed with salivary proteins in sputum, but because the protein content of saliva is small, its contribution to the total protein content of sputum is minimal.

Several plasma proteins appear in the airways as a result of transudation. Some observers contend that some plasma proteins are actively secreted such as albumin, IgA, lactoferrin, lysozyme and amylase. IgA and lysozyme are also considered markers of mucosal gland secretion (3, 14, 20, 21).

In the mucus of a normal person there is less than 1% concentration of nucleic acids (DNA + RNA), which results from discarded cells, oral bacteria, macrophages, *et cetera*. The nucleic acid content increases up to 7 % in infected sputum (22, 23). The concentration is greater in airway mucus than in gastric or cervical mucus. The lipid content in normal mucus is made up of fatty acids only, and ranged from 0.3 to 0.9 μg/mg of mucin (24). According to Carlstedt (24) no triglycerides, cholesterol, phospholipids or glycolipids were found. The lipid content tends to increase in pathologies like chronic obstructive pulmonary disease. It is clear that DNA contributes to the hyperviscosity if mucus is infected, but the role of the lipids in modifying the physical properties of mucus has not been established.

The surfactant factor

The components of the surfactant factor are synthesized, packaged in the endoplasmic reticulum in the type II pneumocytes and stored in the lamellar bodies (LB). From there they are secreted to the airways. Lamellar bodies and tubular myelin (TM) may interact sequentially to form common myelin (CM), which has had previous extracellular interaction with cholesterol (25). The surfactant factor composition includes lipids, proteins and carbohydrates (26).

We may question why the trachea does not contain a secretion composed of all the components that are produced while traveling up the escalator of the bronchial tree. The movement of the mucus blanket in the airways is continuous. The mixture of the different types of secretions produced by the secretory cells is also continuous. Surfactant components could possibly mix with other secretory products, such as those of the Clara cell and other cells while moving up the escalator. Research on beagle dogs and on frogs has demonstrated that mucus moves at varying speeds during different seasons (2, 27).

A possible function of surfactants in the bronchial tree could be to prevent cells or parts of a cell, like the cilia, from being covered by a layer of mucus that would eventually interfere with their normal function. If this is the case, we may suppose that a very thin layer of the surfactant factor would bathe the tips of the cilia. This would favor the interaction of the cilia's claws with the mucus network and propel the mucus blanket so that the mucus does not stick to the cilia.

Yoneda (28) and Morgenroth (29) have demonstrated the presence of a surfactant factor both within and between the sol and gel phases of the mucus layer, as well as common myelin structures within the sol layer. The specific function of these structures in the mucus layer is still not known, but their role may be to facilitate mucus clearability, or cough clearability, by allowing the mucus layer to slide easily and more effectively on top of the cilia.

Another possibility is that instead of lying on top of the periciliary fluid, the surfactant factor could be incorporated biochemically in the mucus structure. In this case, it might not function as a surfactant, but instead coalesce into inclusion bodies, with essentially a neutral effect on mucus properties and function. There may be some other functions of the surfactant factor other than alveolar mechanics such as assisting in nonciliary clearance of exogenous particles, and mucociliary clearance.

There are two possible ways for the alveolar and bronchial secretions to move forward or up the airways' escalator. Alveolar or surfactant secretions generate an upward concentration gradient, resulting in a surface transport that is either steady state or dynamic. The surface film pressure creates a steady

state transport. The dynamic transport may be a result of the alternating compression and decompression of the surface or a consequence of the asymmetric changes occurring in the alveolar area under inspiration and expiration (30). The movement of the pleura may be compared with the barrier movement in a Wilhelmy balance.

If this is the case, it is clear that the composition of the mucus blanket at the level of the trachea is not consistent with the total amount of secretion produced in the airways. Furthermore, if we consider that mucus is moving up from a vast surface area, and the number of secretory cells is increasing as well, there exists a large volume of secretion that must somehow be accounted for.[1] In fact, Jeffery (31) contends that approximately a total volume of 100 ml per day arrives at the larynx, although 10 ml/day might be considered a more conservative estimate in the normal situation, based on a mucus blanket depth of 10 microns and a linear velocity of 10 mm/min.

Therefore we may propose the existence of a very effective system that actively or passively reabsorbs the excess volume and in the same fashion secretes other components. Due to these compositional changes, as well as the considerable volume involved, the mechanism must include absorption and/or recirculation of macromolecules as well as water. If significant transfer of fluid occurs from the alveoli to the conducting airways, the magnitude of reabsorption that would have to occur would be even greater, given the vast surface area of the pulmonary zone.

Mucus in the gastrointestinal system

The gastrointestinal epithelium has a varied morphological appearance along its tract. Folds, villi, crypts, and papillae make the epithelium look different and also may increase the area of absorption and exchange along the

[1]If the tracheal diameter is 1.7 cm, the linear velocity 12 mm/min, and the mucus blanket depth 10 microns, then the mucus flux is 9.2 ml/day. On the other hand, the total volume secreted by the tracheobronchial tree, based on the surface area of each airway generation and its estimated mucus velocity and depth, amounts to some 400 ml/day (32, 33). [See Table II-1].

Table II-1

Total Mucus Production (cm^3/day) by Region

Region	Number	Diameter (cm)	Axial Length (cm)	Total Crossection (cm^2)	Velocity (cm/min)	Depth (cm)	Net Flux (cm^3/day)
Trachea	1	1.7	12	2.3	1.2	.001	9.2
Main bronchi L	1	1.2	5	2.67	0.8	.001	4.3
Main bronchi R	1	1.4	2.5	2.67	0.8	.001	5.0
Lobar bronchi	5	.8	3	2.5	0.8	.001	14.5
Segmental bronchi	18	.5	6	3.6	0.4	.001	16.3
Intrasegm. bronchi	252	.3	2.5	17.9	0.2	.001	68.4
Bronchioles	504	.2	2	16.1	0.1	.0005	22.8
Sec. bronchioles	3024	.1	1.5	24	0.1	.0005	68.4
Term. bronchioles	12096	.01	0.5	47	0.1	.0005	191.5
Total							400.5

Mucus velocity estimates taken from ref 32 (King & Bates, p. 76);
Regional airway geometry based on ref 33 (Hounam).

Net flux = No. x diam. x π x depth x velocity x 60 x 24 = cm^3/day.

tract. The entire epithelium is covered by mucus. Mucus is mainly produced in the goblet cells, as well as in glands. The epithelium is exposed to other types of secretions like hydrochloric acid, pepsin, and enzymes.

The primary function of the gastrointestinal mucus is the protection of the gastrointestinal mucosa (34). Mucus is involved in absorption and lubrication. Mucus can also act as a mixing barrier. The mucus blanket is conceived as a dynamic system that is continuously moving. The mucus layer is constantly under disruption and repair. For mucus to fulfill its function there must exist a dynamic equilibrium, that is, the production of mucus has to be in equilibrium with the destruction of mucus. A variety of components along the gastrointestinal tract, including bile, pepsin and acid can influence and destroy the mucus surface layer. There are additional factors that can alter the physical properties of mucus such as biologically active materials from food, hormonal secretion or enzymes, part of the flora of the intestine and debris from dead mucosal cells. Changes can be of any kind such as in pH and ionic composition.

Gastric mucus protects the surface of the mucosa against acid and pepsin action. To exert this protective function, a difference in pH must exist between the surface of the gastric and duodenal surface of the mucosa and the luminal surface of the mucus layer. It is believed that the mucosa of the stomach secretes bicarbonates and that the hydrochloric acid produced by the stomach lies on top of the layer of mucus which is being mixed. It is then neutralized by the secreted bicarbonate. This will create a pH gradient across the mucus layer. Williams (35) measured a pH gradient in the gastric mucus of a rabbit that ranged from 2.31 pH in the luminal surface to 7.26 on the mucosal surface of the mucus.

It still is not known how the gastric acid and pepsin travel through the mucus layer, nor how the cells of the gastric epithelium are or can be protected from the effects of pepsin and acid. The negatively charged sialic acid and ester sulfate can bind cations and contribute to the barrier property. The barrier property can also be affected when there are changes in the terminal residues of the sugar chains. Mucus in the gastrointestinal tract can stick to the surface of

the cells and in doing so its lubrication property is enhanced.

The mucus properties in the gastrointestinal tract and in some other systems are determined by the composition of the glycoproteins and also by the structural organization of the macromolecules into the three dimensional structure of mucus. Changes occurring in the characteristics of mucus at either the molecular or the supramolecular level will alter the rheological properties and the physiological functions of mucus. There is limited understanding about how the three dimensional structure of the glycoprotein chain can affect the function of the mucus (36).

The gastrointestinal tract in particular is an area where one can see histochemical changes occurring in several pathological entities. These alterations may or may not have anything to do with malignant changes even though they may be found in malignancies. In one area, although there may be no qualitative changes in the glycoproteins, some quantitive changes may be observed in the relationship of the glycoprotein components.

The viscoelastic properties of mucus in the gastrointestinal tract depend on the structure and constituents of the glycoproteins. The main functions of mucus depend as well on the structure and constituents of the glycoproteins. The presence of nucleic acids and free protein may play a role in the rheological properties of the mucus located in the gastrointestinal tract, particularly in the lower section. Glycoproteins are macromolecules which expand as a consequence of the negative charge that causes repulsion among the charged groups. However, Meyer (37) and Litt (38) demonstrated that removal of sialic acid did not significantly affect either the rheological properties of the mucus or the mucociliary transport.

By using proteolytic enzymes and reducing agents in mucus of different species including human gastric mucus, researchers have found the presence of disulphide bridges which contribute to the glycoprotein structure. The breaking or alteration of these bridges in pathological conditions could decrease or even destroy the effectiveness of mucus as a barrier (39).

A common feature is observed in all of the mucus glycoproteins of gastrointestinal origin. Serine and threonine are linked to the carbon at position

one of N-acetylgalactosamine through their hydroxyl groups, similar to the situation in the respiratory tract. The protein core has a dense covering of carbohydrate side chains which may protect it from the effects of enzymatic action. Mucus glycoproteins have a high carbohydrate-protein ratio and a high molecular weight.

Glycoproteins at different gastrointestinal levels vary in the size of the carbohydrate chains and in the proportion of sugar composition. The percentage of glycoproteins between the mucus in the stomach and other parts of the gastrointestinal tract differs. Sixty to seventy percent comes from the stomach whereas the mucus in the colon has less than 10 % of glycoproteins, as demonstrated in pig mucus samples. The remaining percentage in both stomach and colon comes from free protein and nucleic acids (39). The small intestine mucus glycoprotein differs from gastric and colonic glycoprotein in that it has a higher concentration or proportion of both N-acetylgalactosamine and sialic acid which results in an increased negatively-charged composition (34).

There is some controversy over how the oligosaccharide side chains of the glycoproteins are synthesized. Silberberg (ref. 1,.p 56) contends that in the sugar sequence addition "perhaps all the cell really knows and wants to do is to add sugars in a certain preferred order statistically". Other observers like Watkins (40) argue that the sequence of sugar is genetically governed, and that linkages are also genetically determined. If genetics is the answer, then certain enzymes like glycosyltransferases should play an important role. The genetic control concept of oligosaccharide building gains further support when we examine the terminal end of the sugar chains in human gastrointestinal mucus. These terminal ends are similar to those of the sugar chains on the erythrocyte surface.

The terminal sugar chain determines the specificity of the ABO blood groups. In blood group A, gastric mucus glycoproteins end or terminate with N-acetylgalactosamine, whereas in blood group B, these glycoproteins terminate with galactose. In the AB blood group mucus glycoproteins may terminate with either galactose or N-acetylgalactosamine.

The length of the sugars can be altered or modified during biosynthesis by

endogenous or exogenous factors. An endogenous factor may be the presence of hydrolytic glycosidases in the gut. An example of an exogenous factor may be the bacteria of the intestinal flora which produce neuraminidases; these in turn have an effect on the terminal sialic acid of the sugar residue.

Ester sulfate and sialic acid can be found in the glycoprotein of gastrointestinal mucus. Ester sulfate appears more often in an internal position of the chain of the glycoprotein whereas sialic acid always occurs in a terminal area. At the lowest pH values, sialic acid is not fully ionized. This negative charge in the terminal residues may play a role in the repulsion of adjacent negatively-charged carbohydrate chains and may result in both expanding the structure of the molecules and increasing the viscous properties. Ester sulfate and sialic acid may also bind divalent cations such as calcium.

The N-acetylgalactosamine structure changes in some non-malignant processes such as metaplasia of the gastric mucosa. This metaplastic change gives the gastric mucosa the appearance of intestinal mucosa. This process may occur as a result of inflammation of the gastric mucosa. A reduction in the number of the glycoprotein residues is observed. Also, there are some histologic changes. Goblet cells replace oxyntic and peptic cells. In gastric malignant processes, it is also possible to observe some biochemical alterations in addition to the anatomical transformation. There may be a significantly lower content of serine compared with threonine and a quantitative change in the ratio of the monosaccharide units from the normal glycoproteins (the glycoproteins will have more sialic acid). These changes may occur as a consequence of some degradative process or they may be genetically controlled. The following evidence supports the view that these changes are genetically-controlled: 1) there is a quantitative change in the length of the oligosaccharide units; 2) additional transferases may be required; 3) sialylated glycoproteins are present; and 4) there is a lower content of serine.

The transport of the intestinal contents within the intestines is exerted by several factors, in addition to peristalsis (41). Vander (42) contends that a gradient in the frequency of segmentation is the main factor for propelling the chyme. The peristaltic movement of the intestines travels only a short distance.

Disulphide bridges and glycoprotein subunits

The gastric mucus glycoprotein in pigs appears to consist of four subunits joined by disulphide bridges. Each subunit consists of two parts or two regions: 1) a glycosylated region; and 2) a non-glycosylated region. The protein core has a ratio of 3:1 for both regions. Human gastric mucus has a similar structure to pig gastric mucus carbohydrate side chains (39).

The disulphide bridges are located in the non-glycosylated region of the protein core. Disulphide bridges are important for maintaining the macromolecule structure of mucins. Comparing the properties of gastric and small intestinal mucus in pigs, it has been found that the glycoproteins are similar in size but the lengths of the carbohydrate side chains differ. They also differ in how they are charged. The amino acid composition and the number and size of subunits in the polymeric structure vary as well. In spite of these differences, gel formation is similar (43).

Mucus in the genitourinary system

Semen is known to be a complex vehicle, but because of the presence of proteases it is difficult to analyze its glycoprotein content and rheological properties. Otherwise its study could be useful in understanding the secretory defect in males with cystic fibrosis who are known to be infertile because of a blocked *vas deferens*.

Cervical Mucus

Mucus in the female genital tract is produced in several areas such as in the endometrium, fallopian tubes, Bartholin glands, and particularly in the cervical canal, which is the main site of production. Mucus produced in the fallopian tubes, endometrium and cervix mixes to form a more complex kind of mucus. There is little information about the physical properties or the chemical composition of the mucus in the fallopian tubes and in the Bartholin glands other than that the mucus helps in lubricating the entrance to the vagina for coitus (44).

The cervix of the uterus is under the influence of the hormones during the menstrual cycle. Its anatomical properties are affected, such as the reduction in the size of the opening of the entrance, as well as the amount and physical properties of the mucus produced. The epithelium of the cervix is made up of two types of cells: a ciliated cell and a non-ciliated cell that is considered to be a secretory cell (45). The mucus of the cervix may provide several functions such as facilitating or blocking the entrance of the spermatozoa, and protecting the spermatozoa from the hostile environment. It is also probably a source of energy for the sperm and may serve as a filter for normal sperm.

Mucus changes according to the phases of the cycle. In the proliferative phase of the menstrual cycle, mucus becomes watery and abundant whereas in the secretory phase it becomes thicker and less abundant. The physical properties of the mucus glycoproteins change during the ovulatory cycle. There is a thin secretion of mucus at the time of ovulation with low viscoelasticity. During ovulation the spermatozoa can penetrate more readily than in the luteal phase. The mucus plug during pregnancy is similar to the luteal phase mucus.

The amount of genital mucus produced daily, measured as wet weight, varies from 20 to 600 milligrams a day depending on the period of the cycle. It is probable that the dry weight produced does not vary that much. The physical properties of the mucus glycoproteins change during the ovulatory cycle.

The mucus in the cervix is influenced not only by the sex hormones but can be exposed and suffer changes in varying degrees because of several other factors such as reactions to inflammation, infections, local irritations and other exogenous or endogenous processes. Some drugs also can impose changes in the mucus structure (46).

Most of the studies on the variation in cervical mucus during the two main stages of the menstrual cycle have been conducted in bovines (47). The molecular structure of the glycoprotein in bovine cervical mucus is similar to that in humans, although there are certain differences between the two species. In bovines there is more fucose, and there is also a lower percentage of neuraminic acid. Neuraminic acid in bovines is called N-glycolyl. In humans, it is termed N-acetyl (48). It has been proposed that the macromolecules on the

cervical mucin chains are structurally linear (49).

Several authors contend that mucus in the cervical tract is composed of two main components: a gel phase and an aqueous phase. According to Odeblad (50) the aqueous phase could be regarded as cervical plasma. Both the viscoelastic hydrogel and the aqueous phase can suffer changes as a consequence of the effect of the sex hormones that occur naturally through the menstrual cycle or through hormones that are administered externally.

Cervical mucus has several properties that can be seen such as *Spinnbarkeit*, viscoelasticity and the fern phenomenon or arborization, which is probably one of the most sensitive to the variation in the hormonal activity. Arborization has been used for detection of the ovulation period and also for early diagnosis of pregnancy. It also can be used to monitor when ovulation is induced. Arborization can be seen in other mucous secretions as well as in cervical mucus. It occurs in solutions that contain protein or other organic components and electrolytes, particularly sodium chloride. *Spinnbarkeit* and arborization coincide with the shifting of the basal body temperature and also with the maximum receptivity to the sperm and when the penetrability of the sperm is greater.

Some components of the cervical secretions are the immunoglobulins, particularly IgA and IgG, which may be present at certain stages of the cycle. IgM appears to be present during the secretory phase. Some authors believe that certain immunoglobulins, in particular IgA, are produced locally in the cervical sector because they are not found simultaneously in the blood system. Lysozymes are also found in greater amounts than in serum and are located at the mucus-producing end of the cervical system. Lysozymes are capable of bacteriolytic activity against a number of gram positive and certain gram negative micro-organisms. Amylase is another component of the cervical mucus. Because cervical amylase is similar to salivary amylase in its immunological and biochemical properties, there are doubts that it is only produced in the cervix.

Gibbons (51) proposes that glycoproteins solely are responsible for the rheological properties of the mucus, and variations in protein concentration may

not affect the viscoelastic properties. Chantler (52) contends that the rheological properties of the cervical mucus are due to variations in the fluid content of the entire ensemble rather than changes in the degree of chemical cross-linking.

There are two models for the macromolecular arrangement of the mucus glycoprotein. Odeblad (53) suggests that a crosslinked high viscosity component exists as a micelle with bound water in association. A low viscosity phase which contains a freely diffusible water also exists. Lee (54) proposes a random-coiled structured model with no crosslinking. Lee also proposes a molecular arrangement of the human cervical mucus during midcycle. He describes it as "an ensemble of entangled random coiled macromolecules similar to unvulcanized rubber" (54). Therefore the macromolecular structure may differ from the orderly micellar arrangement proposed by Odeblad. Theories on sperm penetration vary according to the model.

Carlstedt (49) proposes a linear flexible structure of the cervical mucins, similar to a random coil that has naked regions alternating with densely-packed regions. Densely-packed oligosaccharide units cover the protein core in the non-naked region. The naked regions may be subject to proteolytic attack. The largest mucins, obtained by breaking the non-covalent interaction may be the basic building blocks. According to Carlstedt, the disulphide bonds play a role in cross linking but do not participate in mucus polymerization.

Glycoproteins in the cervical mucus have a chemical structure composed of a protein backbone or core that has numerous branched oligosaccaride units of different lengths with a three dimensional orientation. The glycoproteins have a high molecular weight and can be degraded into fragments that differ in chemical composition by using reduction or proteolysis. These fragments are called glycopolypeptides. The molecular weight of the proteolysed cervical glycopolypeptide is similar to the glycopolypeptide from ovarian cysts, as well as bronchial, gastric and intestinal mucus (45).

Chantler states that nine to ten monosaccharide units constitute one oligosaccharide unit, and contain five residues: L-fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and N-acetylneuraminic acid.

There is an O-glycosidic linkage between serine or threonine in the protein core and N-acetylgalactosamine at the reducing end of the oligosaccharide chain (50). The linkage is similar in the lung and in the gastrointestinal tract.

The glycoprotein contains about 1000 amino acid residues. The naked peptide which is not glycosylated contains about 200 amino acid residues. This area is susceptible to trypsin digestion. The core region contains approximately 800 amino acid residues that are highly glycosylated. The core has about 200 oligosaccharide side chains containing around ten sugar residues. According to Wolf, (55) the amino acid composition of the mucus glycoprotein core in humans does not appear to change in response to the different hormonal conditions of the menstrual cycle.

Mucus rheology might be affected by changes in the charges that are present as a consequence of the existence of sulfated and carboxyl groups on the oligosaccharide chains of the glycoprotein. There are conflicting views regarding the sialic acid content of the mucus during the menstrual cycle and it is still not known what part of the menstrual cycle has the maximum level. Although some researchers have found that the rheological property of the mucus does not change even with complete removal of the sialic acid, it is known that it is neither affected by the ionic strength nor the pH of the medium. The rheological properties of the cervical mucus are not altered by removal of the terminal neuraminic acid, neither is the mucociliary transport (37). Through lyophilization and rehydration, it has been shown that the content of the glycoproteins of the mucus will have a direct effect on viscoelasticity.

Cervical mucus has either L-fucose or sialic acid at the end of the oligosaccharide chain. In humans we normally find N-acetylneuraminic acid. This seems to be a universal characteristic of the epithelial glycoproteins. The sialic acid to fucose ratio also shows an inverse proportion. This ratio may be altered by three mechanisms according to Chantler: 1) the addition of one residue creates an unfavorable substrate to add to the alternative residue; 2) the Golgi complex may be under negative feedback to add either terminal residue, probably arising from byproducts of the transferase reaction; and 3) there has been a modulation of the termination step.

Conclusion:

Mucus is a unique *materia* that plays an essential role in maintaining a steady and strictly controlled homeostasis in several systems of the body. Mucus is present in the airway system, the gastrointestinal system and the genitourinary system. It is in direct interaction with various types of tissues, for example ciliated cells and non-ciliated cells. Mucus is produced mainly in two types of cells: goblet cells and glandular cells. Other cells such as the Clara cell and the type II pneumocyte, in the lungs, produce a secretion that contributes to give mucus its unique physical properties.

Within the cell there are organelles that may be considered a mucus-producing system, which are responsible for building the macromolecules. We propose to look more closely at this mucus-producing system for early anatomical changes that may explain later events related to malignant and nonmalignant processes. Although it is still controversial, we favor the existence of a genetic control of mucus production.

A complex, common and basic biochemical structure exists in the mucus of the different systems. The mucin macromolecules consists of a proteic core with oligosaccharide units attached to it. This units are built by the same type of cells, and within the cells by a similar microsystem of organelles. Although the main function may vary, mucin macromolecules share a similar structure. We may also expect to see that mucus of the different systems is affected in a similar fashion by a single factor. One important factor to consider in mucus analysis is the stereometric change that may occur in the macromolecular structure as a result of injurious agents. How may this change alter mucus properties?

The complex biochemical composition allows mucus to exert its unique physical properties. Viscosity and elasticity are the physical characteristics considered basic for mucus to function, although there are probably other equally important characteristics, such as adhesivity and spinnability.

There is an optimum range among the physical properties that gives mucus its capacity to behave in a balanced manner even at very different frequencies or rates of displacement. Frequency is important for mucus

clearance, particularly in the airways. Two clearance mechanisms take place in the lungs, bicellular clearance and cough clearance. Low frequency or low amplitude is related to bicellular clearance, while high frequency/high amplitude is more closely related to cough clearance (56). In the gastrointestinal tract, on the other hand, there has to be a balance between the stability of the mucus as a protective layer, and its capacity to permit absorption of nutrient macromolecules. In the cervix, a balance must also exist between the function of mucus as a barrier and its requirement to allow fertilization to take place.

Several factors may disrupt this optimum balance or range and create a change that may be progressive and definite. How long it will take for these changes to become permanent is still an open question. The structural changes that will lead to reversible or irreversible changes in the composition of the mucus are even more debatable. Although much is known about the chemical and physical properties of mucus, there remains much more to be explored.

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Chapter III

TRACHEAL MUCUS RHEOLOGY IN PATIENTS UNDERGOING DIAGNOSTIC BRONCHOSCOPY: INTERRELATIONS WITH SMOKING AND CANCER

Introduction

Mortality due to lung cancer continues to be a major problem in our society. Most of the excess deaths can be directly attributed to tobacco consumption; yet despite years of adverse publicity regarding the link between cancer and cigarette smoking, large segments of our population continue to smoke. Particularly distressing is the fact that many young people take up the habit fully aware of the health hazards, and the rate of tobacco consumption continues to increase in developing countries (1). Unless attitudes change drastically, we can expect that lung cancer and other tobacco-related diseases will continue to be a problem for the foreseeable future.

There have been many studies that have explored the prognostic indicators of the development of lung cancer. Recent attention has been focused on small airway obstruction, and its possible role as a promoting factor (2- 4). Abnormalities in mucociliary clearance, which could relate to both obstruction and cancer, have not received as much attention.

Several years ago Hilding (5) suggested that abnormalities in "ciliary streaming", leading to prolonged retention of carcinogens, could be involved in the etiology of lung cancer. Matthys and co-workers (6) recently showed that patients with chronic bronchitis and cancer had slower mucociliary clearance than patients with comparable lung function but no cancer. They speculated that the discrepancy could be due to differences in the rheological properties of the mucus. One of the aims of our study was to examine this possibility.

A number of studies have indicated that chronic smoking leads to abnormalities in mucus clearance, both in humans (7, 8) and in animals (9). Our own work in a canine model of smoking (10) indicated that mucus rheology is altered in a favorable way by cigarette smoke exposure, perhaps to partially compensate for ciliary dysfunction. This alteration is relatively short-lived, however, and disappears with continued smoke exposure. Our second aim was to see if the mucus from human smokers behaved in a similar fashion.

Materials and methods

Subjects: We chose 43 subjects at random who underwent diagnostic bronchoscopy for various pulmonary abnormalities, excepting subjects with obvious airway infections. The bronchoscopic procedure followed standard guidelines with the exception that no atropine was administered until after the collection of the mucus sample for rheological analysis. Xylocaine was used as required for local anesthesia. The experimental protocol was approved by the University of Alberta Hospital Medical Ethics Committee. Informed consent was obtained from all subjects.

The majority of subjects underwent pulmonary function testing (PFT), bronchoalveolar lavage (BAL), cytology and biopsy, in addition to the bronchoscopy, as part of their diagnostic workup. A detailed smoking history was obtained from all but one subject.

Mucus collection: For mucus collection we used the bronchoscopic technique developed by Jeanneret-Grosjean *et al* (11). As described previously, the hypopharynx was anesthetized by topical application of 4% xylocaine, and a flexible bronchoscope introduced. A cytology brush was placed in direct contact with the tracheal mucosa for 10 to 15 seconds, thus allowing for the collection of several microliters of mucus. The mucus sample was scraped from the brush and immediately covered with paraffin oil. The viscoelastic properties were determined by magnetic microrheometry within one hour of collection. The rheological analysis was carried out without knowledge of the diagnosis. The mucus collection, which took less than one minute in total, preceded any other bronchoscopic procedure. No anticholinergic premedication was given. Most patients required additional intratracheal xylocaine to suppress coughing.

Rheological analysis: The magnetic microrheometer technique (12) consists of introducing a steel sphere, approximately 100 μm, into the mucus, which is then oscillated in a magnetic field produced by alternating current. The displacement of the image of the ball is magnified by a microscope and detected by an optical system. The signals corresponding to ball displacement

and magnetic force are displayed together on an oscilloscope, forming an ellipse, from which viscosity and elasticity can be determined. The method requires only microliter quantities of mucus.

Two viscoelastic parameters were studied: G* (modulus of rigidity or rigidity factor) and tan δ (loss tangent or recoil factor), each measured at a frequency of 1 and 100 rad/s. G* is the vector sum of elasticity and viscosity, and is a measure of the resistance to deformation or rigidity. Tan δ is the ratio of viscous to elastic deformation. A material with high tan δ deforms permanently when subjected to a stress or force; a material with low tan δ recoils or snaps back after the stress is removed. The mucociliary clearability index (MCI), indicating clearability by normalized ciliary function, was computed from G* and tan δ at 1 rad/s, and the cough clearability index (CCI) computed from G* and tan δ at 100 rad/s. The two indices are derived from model studies of clearance (13, 14). Both indices relate negatively with the rigidity factor; MCI also relates negatively with the recoil factor, but CCI relates positively with it. Their respective formula are as follows:

$$MCI = 1.62 - 0.22 \times \log G^*_1 - 0.77 \times \tan \delta_1 \qquad (1)$$

$$CCI = 3.44 - 1.07 \times \log G^*_{100} + 0.89 \times \tan \delta_{100} \qquad (2)$$

Statistical treatment of data: Data are reported as mean ± SD unless otherwise stated. Analysis of variance, using Fisher's Protected Least Significant Difference test, was used for multiple comparisons among groups. Simple comparisons between groups were based on unpaired two-tailed *t* tests. Intrasubject variables were related by linear regression analysis. A value of $p < 0.05$ was considered significant. The statistical analysis was assisted by a personal computer program (Statview 512+, Brainpower Inc, Calabasas, CA).

Results

Samples of mucus were obtained from 43 patients (24 to 79 years, mean age 59±14). The population included 9 nonsmokers, 18 current smokers and 16 exsmokers (> 6 mo.). Twelve of the patients (9 smokers, 3 exsmokers) had lung cancer. One exsmoker had lung carcinoid. The distribution of tumor types was as follows: adenocarcinoma - 4; small cell - 3; large cell - 2; squamous cell - 2; oat cell - 1; carcinoid, typical - 1.

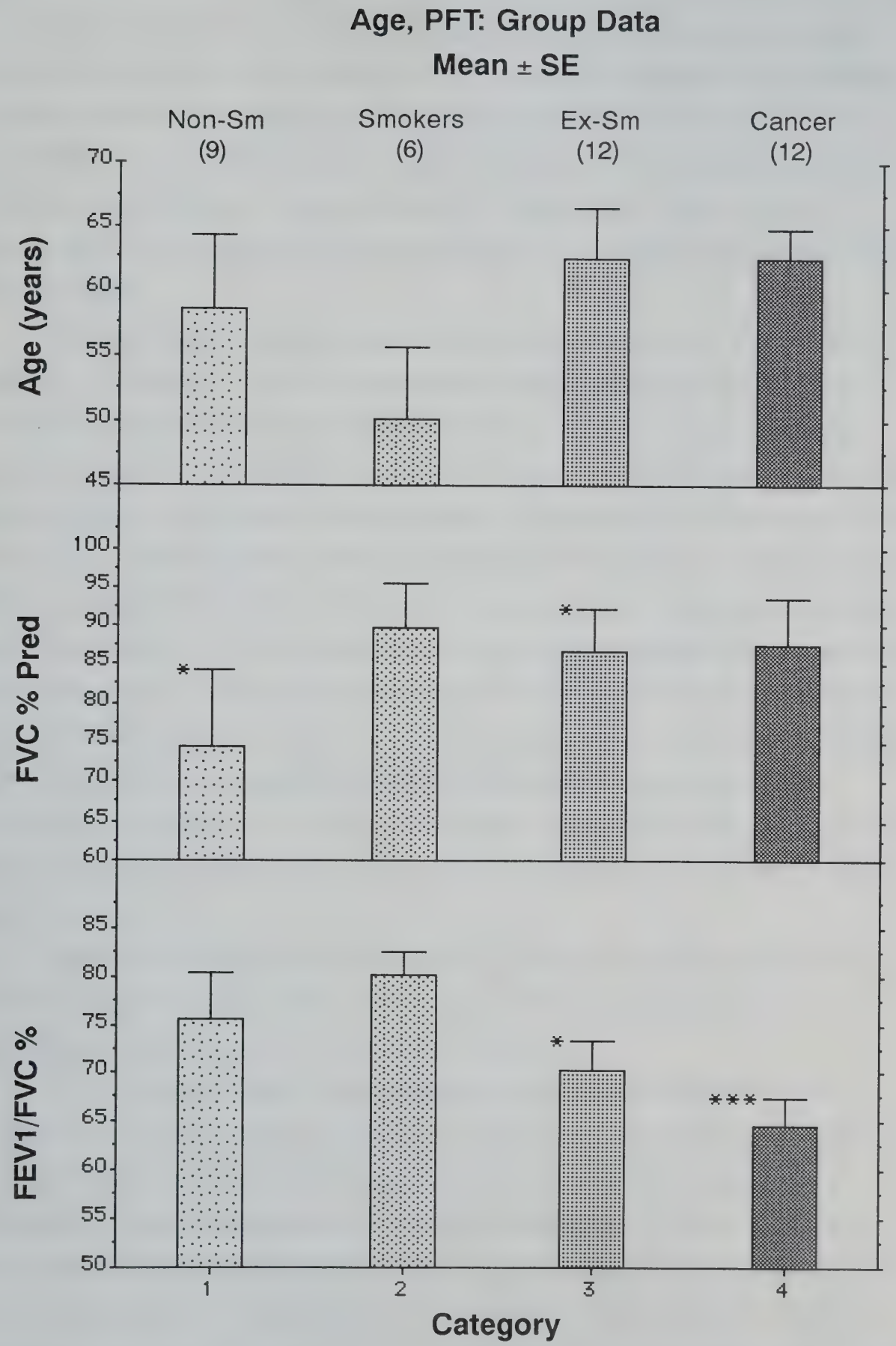
The patients were categorized into 5 groups for statistical analysis as follows: 1) Lifelong nonsmokers without lung cancer (NS, N = 9); 2) Light smokers without lung cancer (LS, N = 4); these included 3 "social smokers" of 1 to 3 cigarettes per day (lifetime exposure 1 to 3 pack years) and one exsmoker with 13 pack-years exposure who had quit smoking 30 years prior to study; 3) Moderate to heavy smokers without lung cancer (HS, N = 6) who currently smoked at least 10 cigarettes per day. Their mean lifetime exposure was 37.4 ± 21.6 pack years; 4) Exsmokers without lung cancer (XS, N = 12) who had smoked at least 10 cigarettes per day and who had quit smoking for at least 6 months at the time of the bronchoscopy. Their mean lifetime exposure was 32.3 ± 14.6 pack years, and the number of years of smoking cessation was 9.2 ± 7.7; 5) Smokers and exsmokers with lung cancer (CA, N = 12). Their mean lifetime exposure was 38.0 ± 13.7 pack years. [This category included 3 exsmokers (7.3 ± 7.8 years of cessation) and 9 smokers, including two individuals who had quit smoking 3 months prior to bronchoscopy and were still categorized as current smokers].

To define a reference group for normal tracheal mucus rheology, data were taken from the study of Jeanneret-Grosjean *et al* (11 - termed the "Montreal study"). Since 2 of the 19 volunteer subjects of the latter study were in fact light smokers, only the data on the remaining 17 were used in defining the normal values of rheological parameters. The age of the Montreal reference group was 28.9 ± 7.3 years; no PFT's were done but all the subjects were healthy.

The age and pulmonary function characteristics of the patient groups are shown in Figure III-1. Although the current smokers without cancer tended to be

Figure III-1

Comparisons of age, forced vital capacity (% pred), and forced expiratory volume in 1 second (% FVC) between four patient groups. [Only 2 of the 4 light smokers had PFT's; thus their data are not presented in this figure. Their age was 50.8 ± 17.2 years]. There was no statistical difference in age among the groups. The reduction in FEV1/FVC for cancer patients with respect to predicted normal values was highly significant ($p < 0.001$). The mean values of FVC for nonsmoker patients and of both FVC and FEV1/FVC for exsmokers without cancer were also significantly reduced ($p < 0.05$). One exsmoker had asthma and rather severe obstruction for a relatively short smoking history, and was excluded from the PFT analysis.


Age, PFT: Group Data
Mean ± SE
Non-Sm (9)
Smokers (6)
Ex-Sm (12)
Cancer (12)
Age (years)
70
65
60
55
50
45
FVC % Pred
100
95
90
85
80
75
70
65
60
*
*
FEV1/FVC %
85
80
75
70
65
60
55
50
*
***
1
2
3
4
Category

younger than the other patient groups, there was no statistical difference in age among the groups. The nonsmoker group had significant restrictive lung disease, as evidenced by a reduced FVC; the cancer patients had significant airway obstruction, indicated by a greatly reduced FEV1/FVC ratio. The exsmokers had mild reductions in both FVC and FEV1/FVC that achieved statistical significance. One exsmoker had asthma and rather severe obstruction for a relatively short smoking history, and was excluded from the PFT analysis.

The rheological findings (mean ± SE) are summarized in Figure III-2, as well as in Table III-1. For the nonsmoker patients, all of the rheology and transport parameters were virtually identical to those found previously for normal volunteers, despite the fact that the majority of the former had restrictive lung disease. For smokers without cancer, the mucus had a lower value of tan δ at 1 rad/s, and exsmokers without cancer had both a low tan δ and a low log G*. The calculated MCI, which is based on mucus transport measured on the *in vitro* mucus-depleted frog palate model (13), would indicate rapid clearance for the mucus from smokers without cancer and even faster clearance values for the exsmokers without cancer. Tracheal mucus from smokers and exsmokers with cancer was not significantly different from either nonsmoker group (Montreal or Edmonton). However, the mucus from cancer patients had a lower MCI than that from the other smokers and exsmokers, mainly due to a higher tan δ.

Detailed anthropometric information, pulmonary function data, smoking history, diagnostic information, and the complete results of the mucus rheological analysis are included in the Appendix D.

The viscoelastic parameters appear to depend on lifetime tobacco consumption (pack years), as far as one can tell in a cross-sectional study. For log G*, there appears to be a temporary advantage favoring mucociliary clearance, which disappears with continued smoking. This is indicated in Figure III-3, where it can be seen that in the early stages of smoking, there is a marked decrease in rigidity (G*) of mucus, with a clear tendency to return to normal or even higher values over a time scale of about 40 pack years. We saw

Figure III-2

Comparisons of log G* (rigidity factor), at low frequency only, and tan δ (recoil factor), at low and high frequency, between the five patient groups and the Montreal reference group (healthy nonsmoking volunteers). Rigidity (G*) data at high frequency are not shown since their logarithmic variation is very similar to that at low frequency. Low frequency data relate to better with mucociliary clearability, while high frequency data relate more directly with cough clearability. The asterisks indicate significant differences with respect to the normal population: * $p < 0.05$, ** $p < 0.01$; the double dagger indicates a difference ($p < 0.05$) with respect to the combined data for smokers and exsmokers without cancer.

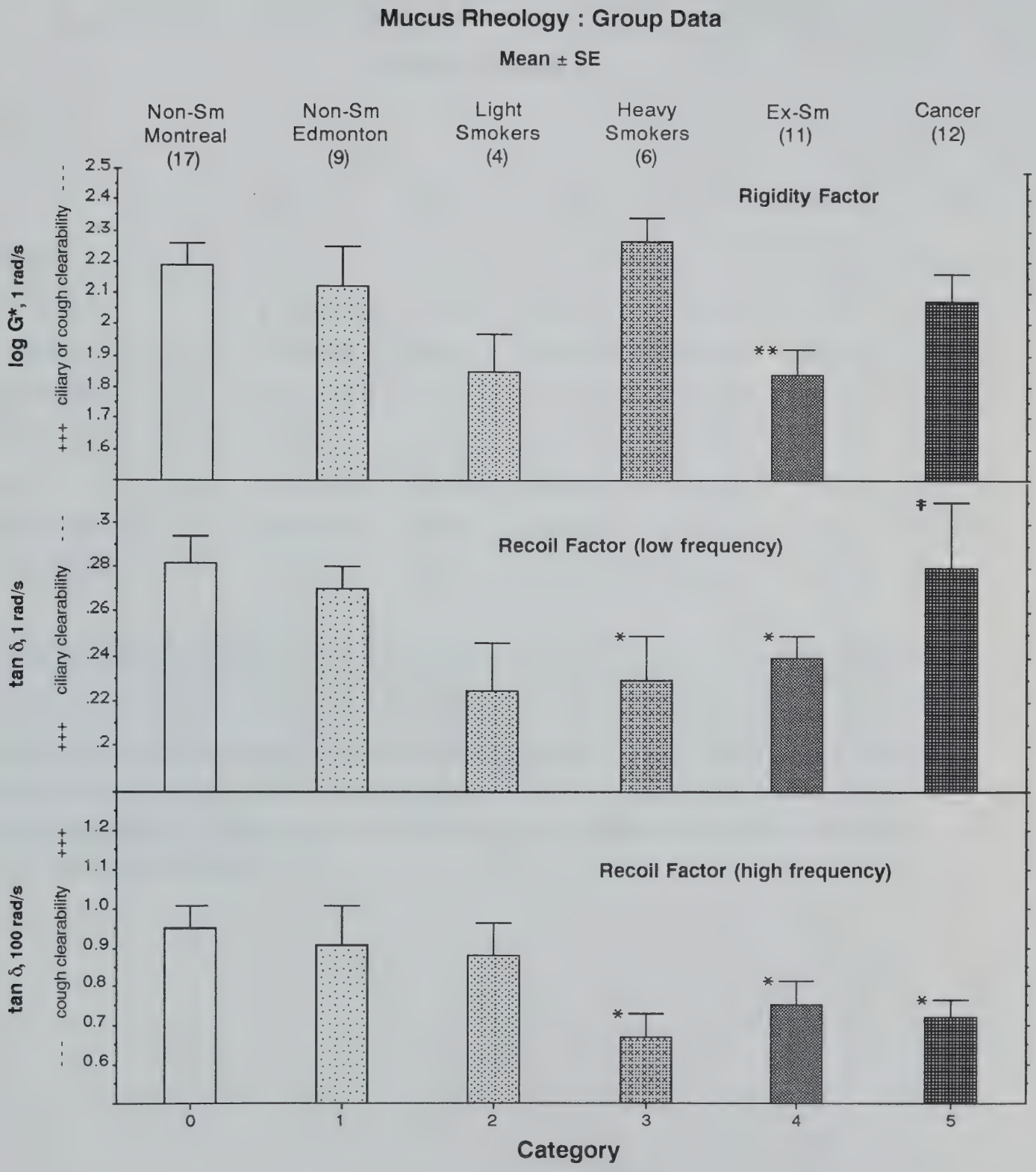

Mucus Rheology : Group Data
Mean ± SE
Non-Sm Montreal (17)
Non-Sm Edmonton (9)
Light Smokers (4)
Heavy Smokers (6)
Ex-Sm (11)
Cancer (12)
log G*, 1 rad/s
+++ ciliary or cough clearability ---
Rigidity Factor
tan δ, 1 rad/s
+++ ciliary clearability ---
Recoil Factor (low frequency)
tan δ, 100 rad/s
--- cough clearability +++
Recoil Factor (high frequency)
Category

TABLE III-1

Summary of Rheological and Transport Parameters (mean ± SD)

	N	log G*,1	tan δ,1	MCI	log G*,100	tan δ,100	CCI
NS(Mtl)	17	2.19±.32	.28±.05	.91±.08	2.58±.33	.95±.26	1.52±.51
NS(Edm)	9	2.12±.40	.27±.03	.94±.09	2.55±.33	.91±.29	1.52±.55
LS(Edm)	4	1.85±.25	.23±.04*	1.03±.07*	2.18±.24*	.88±.17	1.89±.34
HS(w/oCa)	6	2.27±.18	.23±.04*	.94±.05	2.56±.12	.67±.14*	1.30±.23
XS(w/oCa)	11	1.84±.27**	.24±.04*	1.03±.05***	2.16±.24	.75±.21*	1.78±.38
Ca	12	2.07±.32	.28±.06†	.93±.10	2.37±.31	.72±.16*	1.55±.36
HS+XS(w/oCa)	17	1.99±.32	.23±.04	1.00±.07	2.30±.28	.72±.19	1.62±.40
HS+XS(Obstr)	8	1.93±.31	.25±.04	1.00±.07	2.30±.30	.73±.23	1.64±.42

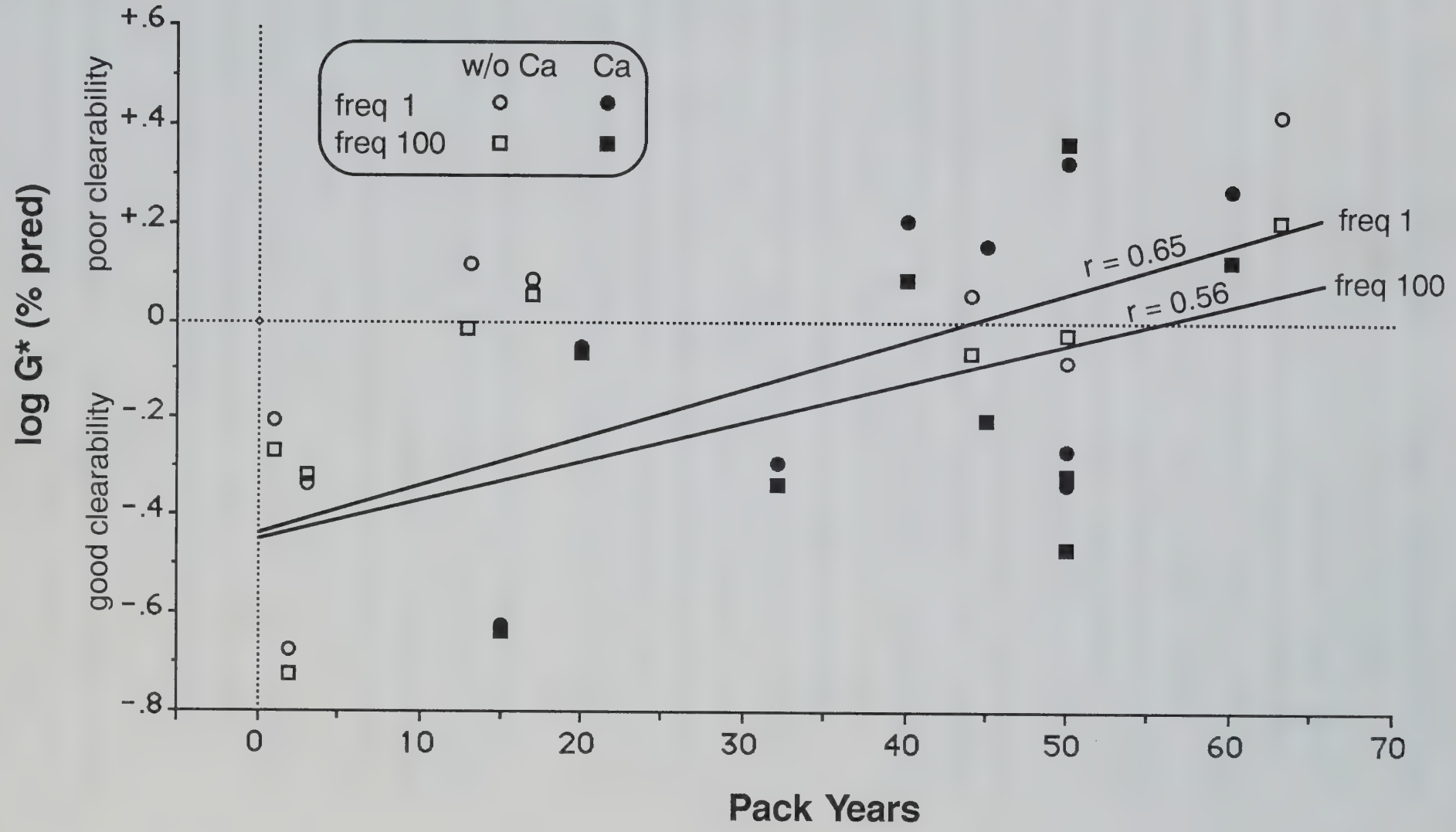
NS(Mtl): healthy nonsmokers (Montreal study, ref. 11); NS(Edm): nonsmoker patients (present study); LS: light smokers (< 5 cig/day or long-time Ex); HS(w/oCa): heavy smokers without cancer; XS(w/oCa): exsmokers without cancer; Ca: smokers and exsmokers with cancer; HS+XS(Obstr): smokers and exsmokers without cancer but with low FEV1/FVC. The asterisks indicate significant differences with respect to the normal population: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; the dagger (†) indicates a difference ($p < 0.05$) with respect to the combined data for smokers and exsmokers without cancer.

Figure III-3

Rigidity factor G* (viscosity+elasticity) of tracheal mucus from current smoker patients with and without lung cancer *vs.* lifetime tobacco consumption (pack years). Rigidity data are expressed as a fraction of non-smoking control and plotted logarithmically. The solid lines are the lines of best fit for smokers without cancer; the slopes are significantly different from zero ($p = 0.007$ at 1 rad/s, $p = 0.023$ at 100 rad/s). About half of the cancer patients follow these regressions, while the others have mucus that is more rigid, less easily clearable for their smoking history. The data for exsmokers are not plotted in this figure, but they follow a similar pattern, i.e. lower than normal G* for modest lifetime exposure and normal or supranormal for more prolonged exposures.

Rigidity Factor *vs.* Tobacco Consumption

Current Smokers Only



a similar pattern develop in smoking dogs on a greatly compressed time scale (10). Presumably, this represents a natural protective response of the mucociliary system to deal with the developing epithelial injury. About half of the cancer patients appear not to have developed this protective response.

The apparent rheological advantage to smoking (i.e. the mucus becoming more easily clearable by ciliary action) may be illusory, as well as being temporal, because it is accompanied by a progressive disadvantage in cough clearability. This is indicated by the data in Figure III-4, which shows a significant fall in the high frequency recoil component, tan δ at 100 rad/s. According to the rheological findings, the predicted clearability of the mucus by coughing decreases with tobacco consumption in all smokers, with or without cancer; the deficit is somewhat accentuated in those who have lung cancer, but not significantly so. The decline in cough clearability with pack years of exposure is much more rapid than the decline in mucociliary clearability.

Furthermore there is evidence that actual mucociliary clearance rates are depressed even in asymptomatic smokers (7, 8), possibly because of the observed ciliostatic effects of tobacco smoke inhalation (15) or due to ciliary exfoliation that has not yet repaired itself (16). This would tend to negate any "advantage" in the rheologic characteristics of respiratory mucus in these patients.

Additional observations concerning the effects of time or age on mucus rheology include the following: 1) there is no apparent age dependence among non-smoking subjects for log G* or for tan δ, although tan δ at 100 rad/s showed a downward trend, i.e. in the direction of impaired cough clearability, but with a lesser slope than the pack year dependence seen in smokers. This is illustrated in Figure III-5. And 2) there is no apparent return to normal values of rheological parameters with smoking cessation, even after 15 to 20 years. These latter conclusions are tentative, and need to be confirmed by further experiments.

The differences in rheology between cancer patients and smokers without cancer persist even when matched for obstruction. This is evidenced by the fact that the smokers and exsmokers without cancer, who have the greatest degree

Figure III-4

Recoil factor tan δ (viscosity/elasticity), expressed as a fraction of nonsmoking control, for tracheal mucus from current smoker patients with and without lung cancer *vs.* tobacco consumption. The dashed lines are the regressions for both groups at each frequency. The deviations from control at 100 rad/s are significant ($p = 0.001$). The latter result implies a poor cough clearability in long-term smokers because the mucus is abnormally elastic (low tan δ) at high frequency, at which point it should be deformable. The data for exsmokers are not plotted in this figure, but they follow a similar pattern to that observed for current smokers, i.e. lower than normal tan δ at 1 rad/s for exsmokers without cancer and a progressive fall in tan δ at 100 rad/s with increasing lifetime exposure.

Recoil Factor *vs.* Tobacco Consumption

Current Smokers Only

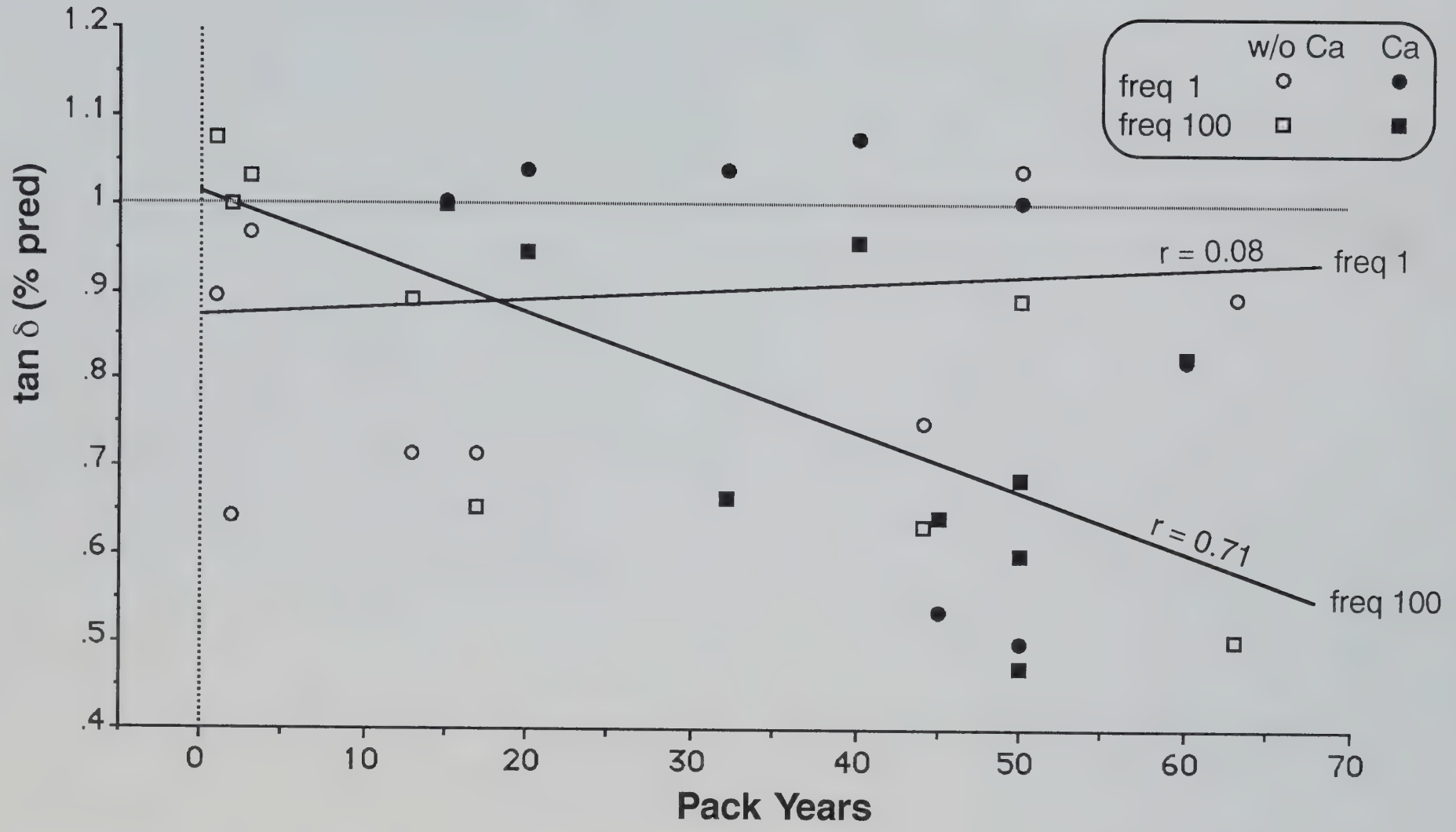
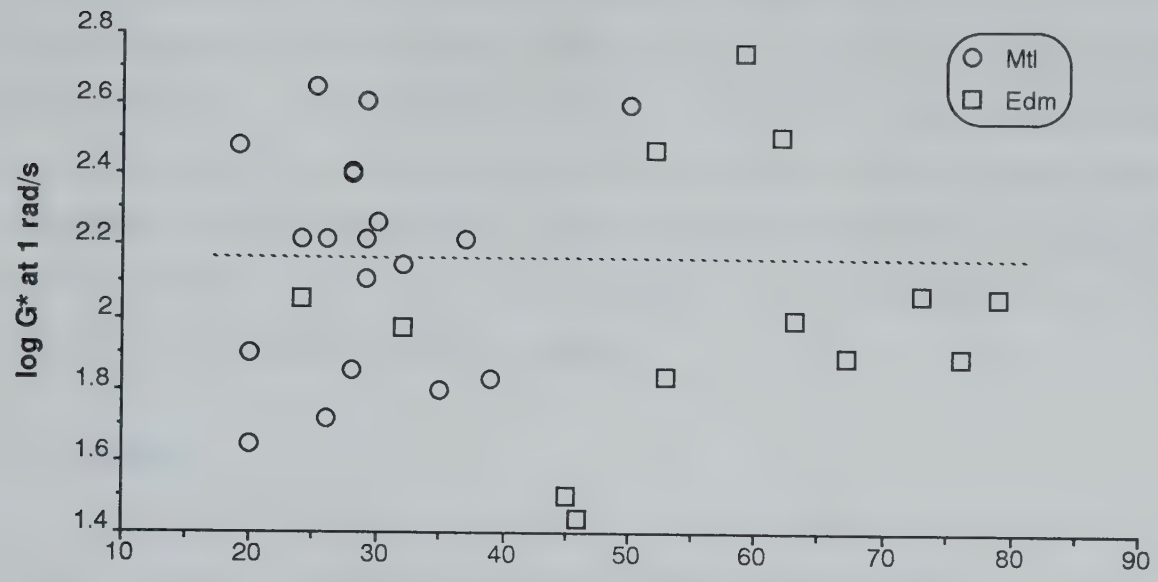
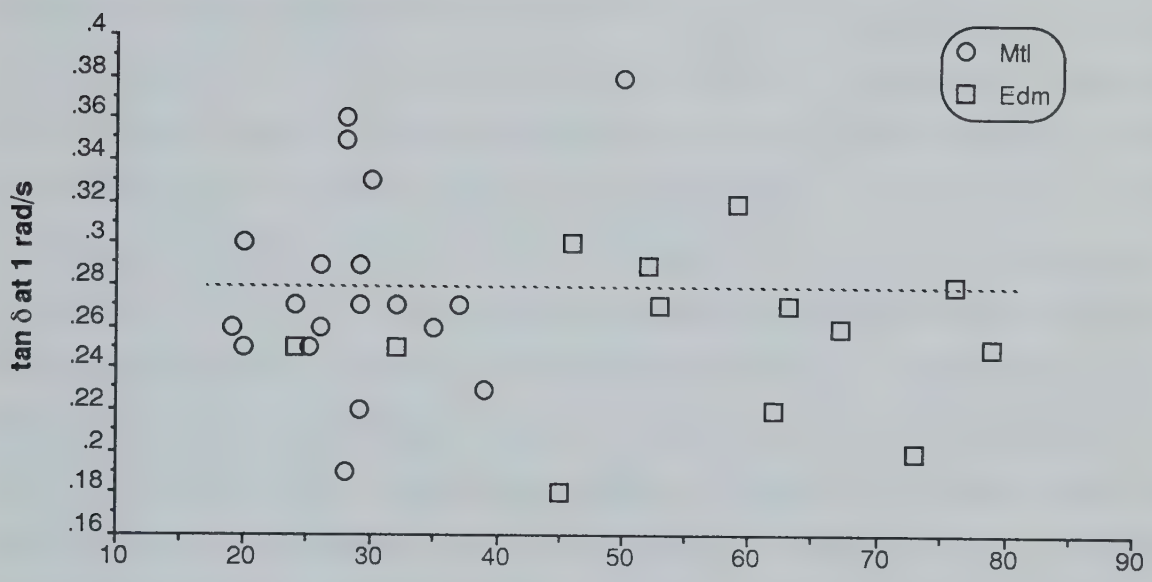


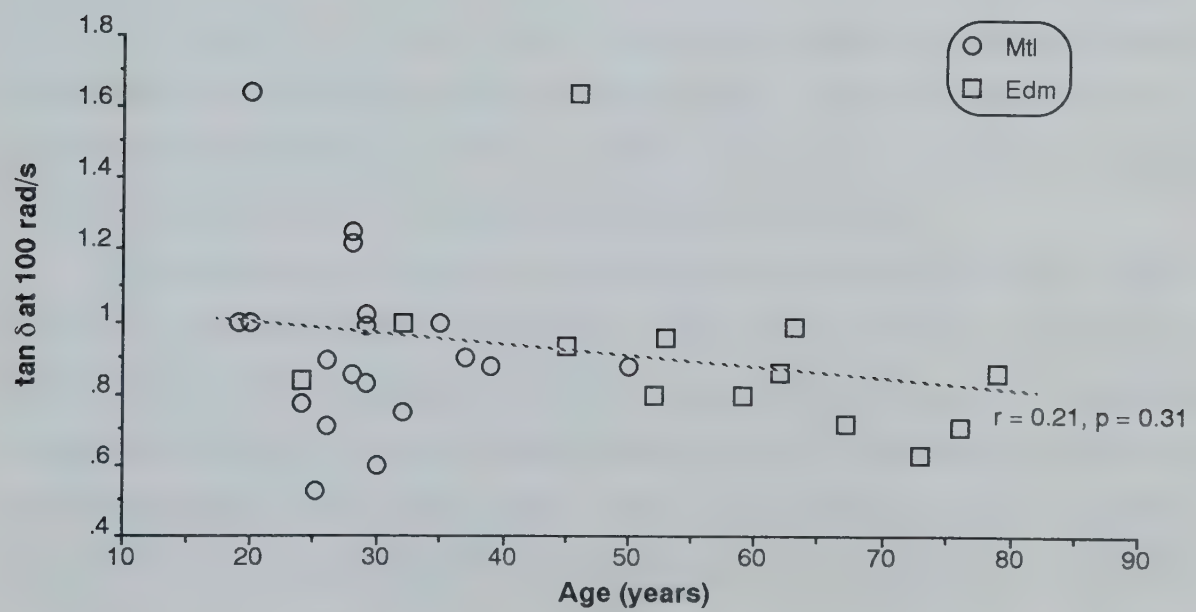
Figure III-5

Rigidity factor log G* at 1 rad/s and recoil factor tan δ at 1 and 100 rad/s for nonsmokers as a function of age. Data for both nonsmoker populations (Montreal volunteers and Edmonton bronchoscopy patients) are included. None of the age correlations is statistically significant. The variation in rigidity factor at 100 rad/s was similar to that at 1 rad/s ($r = 0.07$, NS).

Age Dependence of Mucus Rheology in Nonsmokers







of airway obstruction as measured by the FEV1/FVC ratio, show the same kind of differences in mucus rheology with the cancer group as do those with the least obstruction. These data are shown in Table III-1. Correlations between mucus rheology and airway obstruction in our study were extremely weak, although as noted in Figure III-6, it is interesting that the trends between MCI and CCI and FEV1/FVC are of opposite sign for the cancer patients and the smokers and exsmokers without cancer.

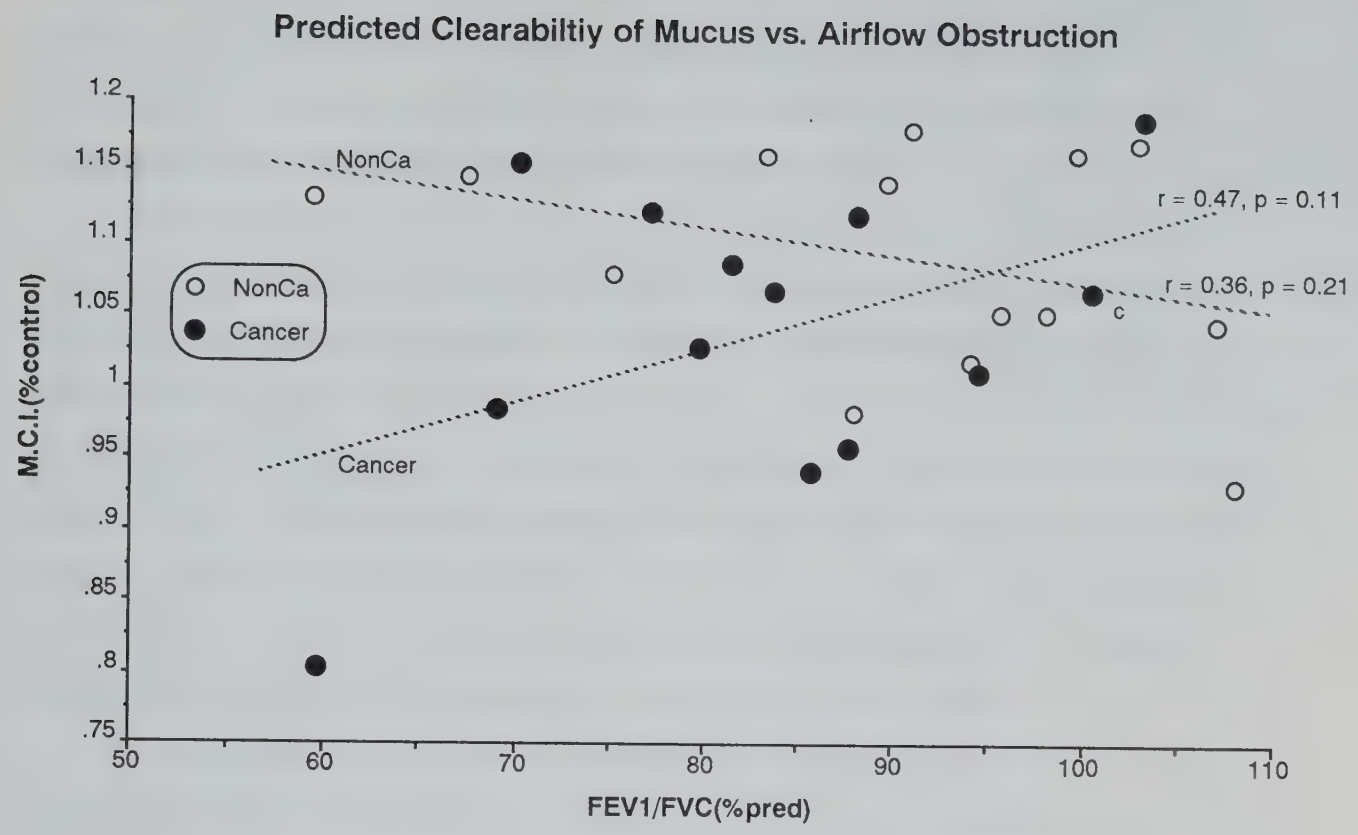
Discussion

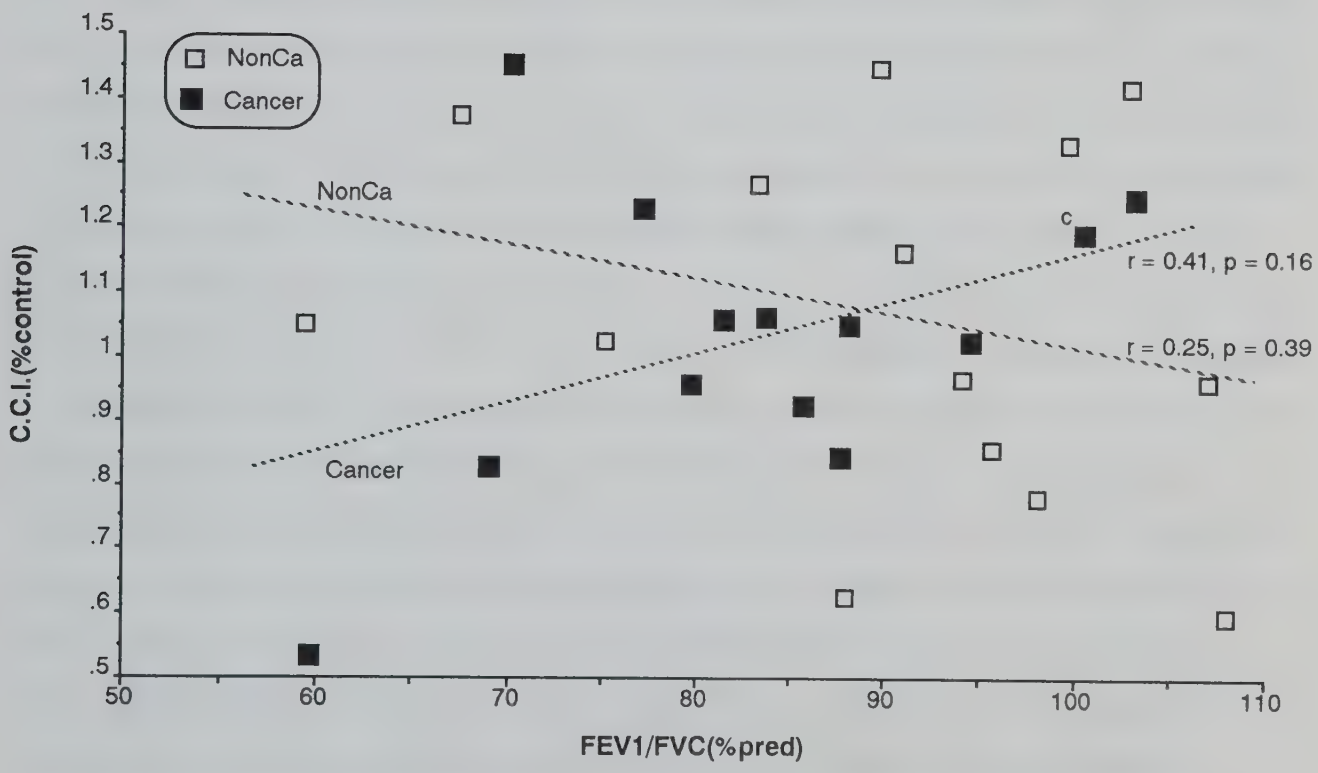
Smokers in this study had a mucus with a lower tan δ at 1 rad/s. This makes the mucus easier to be transported by cilia because the mucus dissipates less mechanical energy than normal, i.e. the energy input of the cilia is more efficiently transformed into forward motion. The mucus of exsmokers had an even better ciliary transportability due not only to a low tan δ, but also to a lower log G* (more deformation for a given force). The alterations in mucus rheology are exposure-dependent – the apparent benefit of smoking on mucociliary clearability has a relatively short life, and disappears in those who continue to smoke. We saw a similar pattern develop in dogs exposed to cigarette smoke even though they had a much shorter period of smoke exposure history (10). We also saw similar rheological changes in our earlier studies of smoke-exposed rats (17) and SO_2-exposed dogs (18). Although the changes in mucus in smokers and exsmokers appear to favor mucociliary clearance because of a lower tan δ, this result should not be misinterpreted as advice to take up smoking, even in the short term, because model studies dealing with cough clearance have shown that although a low tan δ is good for mucociliary clearance, it is bad for cough clearance (14).

The fact that some aspects of mucus rheology (G* in particular) apparently return to normal after extended periods of smoking does not indicate that the mucus of long-term smokers is normal. The studies in dogs (10) showed that the early response to smoking was characterized by a simultaneous fall in both galactose content (as a glycoprotein marker) and solids content, correlating with the fall in G* and suggesting a dilution effect as the principal early response to

Figure III-6

Predicted mucociliary clearability (Eq. 1) and cough clearability (Eq. 2) of mucus from smokers and exsmokers (moderate to heavy tobacco consumption) as a function of FEV1/FVC (%pred). Filled symbols represent cancer patients. The patient with carcinoid is indicated by "c". The dashed lines indicate the best-fit relationships (all non-significant) between the two indices of clearability and FEV1/FVC for the two groups.


Predicted Clearabiltiy of Mucus vs. Airflow Obstruction
M.C.I.(%control)
1.2
1.15
1.1
1.05
1
.95
.9
.85
.8
.75
NonCa
Cancer
r = 0.47, p = 0.11
r = 0.36, p = 0.21
c
50
60
70
80
90
100
110
FEV1/FVC(%pred)


C.C.I.(%control)
1.5
1.4
1.3
1.2
1.1
1
.9
.8
.7
.6
.5
NonCa
Cancer
c
r = 0.41, p = 0.16
r = 0.25, p = 0.39
50
60
70
80
90
100
110
FEV1/FVC(%pred)

the airway insult. The return to "normalcy" of rheological values in the face of continued smoke exposure was not accompanied by a comparable return of galactose concentration, however, suggesting that a different form of glycoprotein was now being secreted. We speculate that a similar type of biphasic response to smoking may occur in humans.

Smokers and exsmokers who develop lung cancer do not display a significant difference in mucus rheology with respect to either group of nonsmokers (patients or healthy volunteers). Compared with smokers and exsmokers without lung cancer, however, the mucus of cancer patients exhibits a significantly decreased mucociliary transportability, mainly due to a higher value of tan δ. Subjects who develop lung cancer seem to fall into two distinct groups. About half of them appear to develop a so-called "normal response to smoking", and the other group shows an abnormal response, in that they develop a mucus that is less easily clearable by ciliary action.

According to model studies, the rheological properties of mucus are frequency dependent for both mucociliary and cough clearability. Low frequency viscoelasticity appears to relate better with MCI, while high frequency is more closely related to CCI. When we plotted and compared the normalized results of tan δ of all smoking groups at both frequencies, the analysis revealed a surprising type of mucus behavior as a consequence of smoking (Figure III-4). At low frequency, smokers and exsmokers with or without lung cancer have a slight to modest deviation with respect to normal. At high frequency, smokers and exsmokers with or without lung cancer both diverge with respect to normal, the divergence being more pronounced in the case of subjects with lung cancer. These results imply that the mucus of subjects with cancer is particularly inappropriate for cough clearance because it has a low tan δ at high frequency, so it will resist permanent deformation during the cough, and has a high tan δ at low frequency, so that it will tend to flow back after the cough is completed. From these observations, we could suggest that in smokers and exsmokers the wider the angle of divergence, the higher the risk to develop lung cancer.

There were four patients categorized as "light smokers", three current smokers of 1-3 cigarettes per day, and one exsmoker whose years of cessation

greatly exceeded his years of exposure. These individuals were tentatively categorized as nonsmokers, consistent with other categorization procedures. After close examination of the data, however, it was decided to treat them as a separate category because of the distinct nature of their mucus rheology. Correspondingly, the reference data from the earlier study of the Montreal group (11) were re-examined, and 2 of the 19 volunteers who fitted the category of light smokers (current smokers of < 5 cigarettes per day) were also excluded from that data set for the purposes of defining normal rheological values. [The rheological data from the two Montreal light smokers fitted closely with that of the Edmonton light smoker group, i.e. lower than normal G* and tan δ, higher MCI].

The findings in exsmokers – the apparent failure to return to control values of mucus rheology for at least 15 years of smoking cessation – are rather surprising. This suggests that alterations in the rheological characteristics of mucus, at least after prolonged smoke exposure, are permanent. Camner *et al* (19) found that the mucociliary clearance rate in smokers improved after 3 months of smoking cessation. Although we do not have serial data, our study would not disagree with these findings, in that the mucus in the exsmoker group (without cancer) was much more favorable for ciliary clearance than that of current smokers.

The patient with lung carcinoid had mucus that was more characteristic of the cancer patients than those without. This patient, an exsmoker (40 pack years, 5 years of cessation) had mucus with an elevated value (0.32) of tan δ at 1 rad/s and a very low value (0.47) at 100 rad/s, given the type of abnormal frequency dependence that was most commonly seen in the cancer group. Two of the cancer patients also showed mucus recoil properties that were highly unusual in that tan δ at 1 rad/s was extremely low (0.14, 0.15), lower than any samples obtained from any non-cancer patient or volunteer, and indeed lower than any sample obtained from dog trachea (10). This hyperelastic mucus is inappropriate for both ciliary and cough clearance, since it is below the optimal value of tan δ for frog palate clearance (0.18 to 0.19: unpublished data). The present data is insufficient to make general conclusions concerning mucus

structure in specific classes of cancer. We also cannot yet determine if studies of the mucus before, during and after treatment will reflect the possible benefit of specific treatment.

Our findings do not disagree with those of recent studies relating the risk for lung cancer and airways obstruction. Indeed, our very small cancer population was more obstructed than our non-cancer population. We do suggest, however, that long-term abnormalities in mucus rheology, and by implication prolonged retention of carcinogens, can represent a possible pathway to the development of lung cancer. Whether the link involves a direct pathway or one that includes obstruction is still an open question, we feel, and needs to be investigated further.

In summary, the mucus of nonsmoker patients, even with restrictive lung disease, has rheological properties that are indistinguishable from the mucus of younger healthy non-smokers. Airways respond to the insult produced by smoking with "protective" changes in mucus properties. These changes are relatively long lasting, but eventually disappear with continued smoking. Lung cancer patients have mucus that is inappropriate for both mucociliary clearance and cough clearance. These results are consistent with the initial hypothesis, that long-term abnormalities in mucus clearance, leading to prolonged retention of carcinogens, can contribute to the etiology of lung cancer.

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Chapter IV

BRONCHIAL MUCUS RHEOLOGY IN LUNG CANCER

Introduction

A number of studies have indicated that smoking leads to abnormalities in mucus clearance, both in humans (1, 2) and in animals (3). Our own work in a canine model of smoking (4) indicated that mucus rheology is altered in a favorable way by cigarette smoke exposure, perhaps to partially compensate for ciliary dysfunction. This rheological alteration is relatively short-lived, however, and disappears with continued smoke exposure. With longer exposure, there was an indication (i.e. lowered galactose content of the mucus) that biochemical changes occur in the mucous glycoprotein, perhaps indicating a form of adaptation in the face of the continued and progressive exposure.

Our previous study (5), in which we collected and analyzed tracheal mucus from patients undergoing diagnostic bronchoscopy, revealed a similar phenomenon. There seemed to be an apparent "advantage" to smoking, in that the mucus produced was less rigid and more easily cleared by ciliary action. This advantage disappeared, however, with continued exposure to tobacco smoke, and the mucus became progressively more difficult to clear by coughing.

Hilding (6), suggested that abnormalities in ciliary streaming, leading to prolonged retention of carcinogens, could be involved in the etiology of lung cancer. Matthys (7) more recently described that patients with chronic bronchitis and cancer had slower mucociliary clearance than patients with comparable lung function but no cancer. They speculated that the discrepancy could be due to differences in the rheological properties of the mucus.

Our first bronchoscopic study (5) revealed that, in patients with various degrees of restrictive pulmonary disease who have not been exposed to cigarette smoke, the rheological properties of their mucus have practically no alteration. Also we found that there were differences in mucus rheology between smokers with and without symptoms, and in smokers with lung cancer and those without, consistent with the hypothesis that poor clearance of carcinogens could contribute to the development of lung cancer. Although smokers were easily distinguished from nonsmokers on the basis of mucus rheology, the differences between smokers with and without cancer were subtle,

at least until the later stages. Thus to improve the specificity of our analysis, in view of the fact that lung cancer is usually isolated to one lung, we decided to collect mucus from the lung with the lesion and to compare it with the mucus collected from the contralateral lung.

Materials and methods

Subjects: Forty-one subjects who underwent diagnostic bronchoscopy for various pulmonary abnormalities were studied. Subjects with clinical signs and symptoms of acute or chronic gross airway infections, i.e. purulent sputum, fever, etc., were excluded. The bronchoscopic procedure followed standard guidelines with the exception that no atropine was administered until after the collection of the mucus sample for rheological analysis. Topical xylocaine was used as required for local anesthesia. The experimental protocol was approved by the University of Alberta Hospital Medical Ethics Committee. Informed consent was obtained from all subjects.

The majority of subjects underwent pulmonary function testing, bronchoalveolar lavage, cytology and biopsy, in addition to the bronchoscopy, as part of their diagnostic workup. A detailed smoking history was obtained from all subjects. A supplementary table indicating all of the anthropometric and rheological data on these patients is provided in the Appendix E.

Mucus collection: For mucus collection, we modified the bronchoscopic technique originally developed by Jeanneret-Grosjean *et al* (8). The hypopharynx was anesthetized by topical application of 4% xylocaine without epinephrine, and a flexible bronchoscope was introduced. A cytology brush was placed in direct contact with the tracheal mucosa for about 20 seconds, thus allowing for the collection of several microliters of mucus. The modification for the present study was to collect a sample of mucus from each mainstem bronchus. The site of the first collection (R *vs.* L) was varied. The mucus collection, which took less than five minutes in total, preceded any other bronchoscopic procedure. No anticholinergic premedication was given. Most of the patients required additional xylocaine to suppress coughing, thereby

preventing airway trauma and bleeding from the sampling site.

As in previous studies, the mucus samples were scraped from the brush and immediately covered with paraffin oil. The viscoelastic properties were determined by magnetic microrheometry within one hour of collection. The mucus collection and rheological analysis were carried out without knowledge of the clinical history, diagnosis, site of collection or the order of collection.

Rheological analysis: The magnetic microrheometer technique (9) consists of introducing a steel sphere, approximately. 100 μm, into the mucus, which is then oscillated in a magnetic field produced by alternating current. The displacement of the image of the ball is magnified by a microscope and detected by an optical system. The signals corresponding to ball displacement and magnetic force are displayed together on an oscilloscope, forming an ellipse corresponding to strain *vs.* stress, from which viscosity and elasticity can be determined. The method requires only microliter quantities of mucus.

Two viscoelastic parameters were studied: G* (modulus of rigidity or rigidity factor) and tan δ (loss tangent or recoil factor), each measured at a frequency of 1 and 100 rad/s. G* is the vector sum of elasticity and viscosity, and is a measure of the resistance to deformation or rigidity. Tan δ is the ratio of viscous to elastic deformation. A material with high tan δ deforms permanently when subjected to a stress or force; a material with low tan δ recoils or snaps back after the stress is removed. The mucociliary clearability index (MCI), indicating clearability by normalized ciliary function, was computed from G* and tan δ at 1 rad/s, and the cough clearability index (CCI) computed from G* and tan δ at 100 rad/s. The two indices are derived from model studies of clearance (10, 11). Both indices relate negatively with the rigidity factor; MCI also relates negatively with the recoil factor, but CCI relates positively with it. Their respective formulas are as follows:

$$MCI = 1.62 - 0.22 \times \log G^*_1 - 0.77 \times \tan \delta_1 \qquad (1)$$

$$CCI = 3.44 - 1.07 \times \log G^*_{100} + 0.89 \times \tan \delta_{100} \qquad (2)$$

Other mucus analysis: The frog palate assay, a direct bioassay of mucociliary transportability (12), was carried out on aliquots of the mucus. The results of this assay are reported as the normalized frog palate transport rate (NFPTR). The samples were also weighed, and where sufficient quantity was available (wet wt. > 2 mg), they were evaporated to dryness (microwave oven, 20 minutes at 650 watts) to determine % solids content.

Statistical treatment of data: Data are reported as mean ± SD unless otherwise stated. Inter-site comparisons were based on paired two-tailed *t* tests. A value of $p < 0.05$ was considered significant. The statistical analysis was assisted by a personal computer program (Statview II, Brainpower Inc., Calabasas, CA).

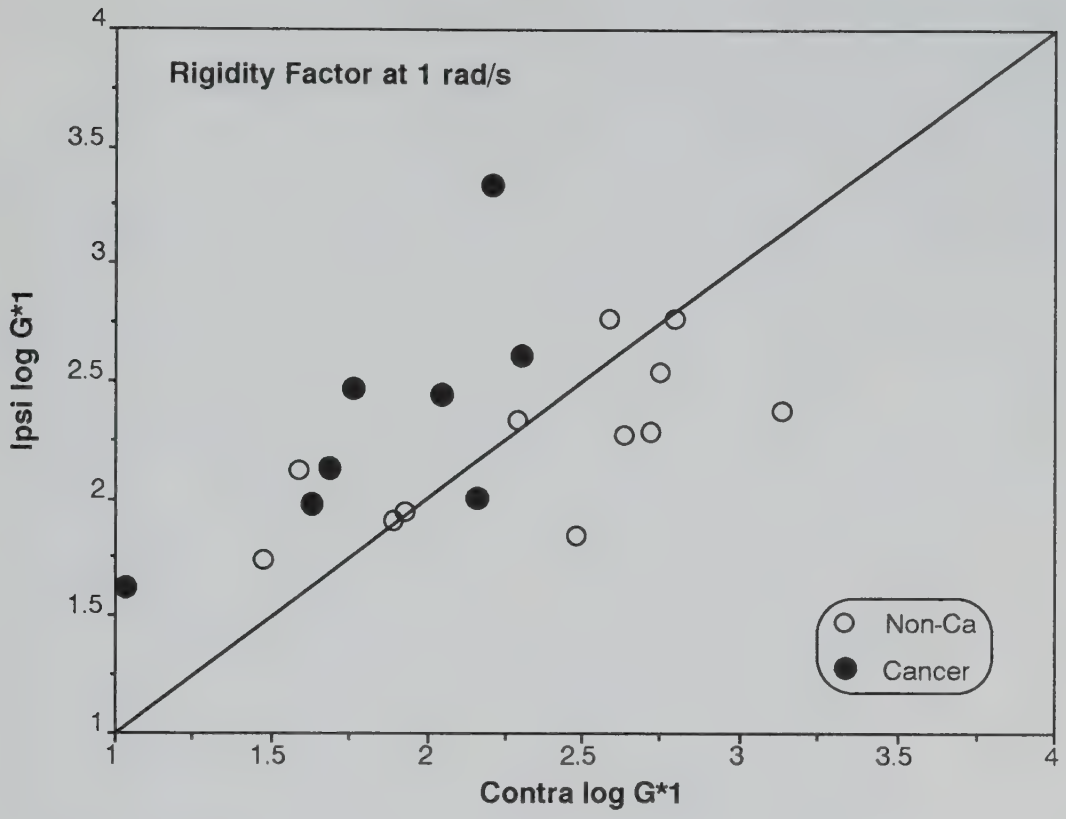
Results

Paired samples of mucus suitable for rheological analysis were obtained from 38 of the 41 patients. These 38 patients included 16 nonsmokers, 14 current smokers and 8 exsmokers (> 1 year); their mean age was 59.8±12.6 years. Twenty of the 38 patients had a radiologically definable lesion localized to one lung. Following the analysis of the data provided by the bronchoscopic procedure or the pathological exam of the biopsies taken, 6 of these 20 patients (4 smokers, 1 exsmoker, 1 nonsmoker) were found to have lung cancer. During the subsequent two years, two more patients (both smokers) were found to have lung cancer, on the basis of followup diagnostic procedures, making a total of 8 patients with lung cancer.

The rheological findings are summarized in Figures IV-1 and IV-2, which show the rigidity factor and the recoil factor for the mucus determined at two different frequencies. For the 8 patients with cancer, the rigidity factor, log G*, was generally greater in the lung with cancer than in the contralateral one. This was true both at low frequency ($p = 0.008$, Figure IV-1) and at high frequency ($p = 0.04$, Figure IV-2). The recoil factor at low frequency (Figure IV-1B) did not distinguish cancer patients from those without cancer, but the same factor (recoil) at high frequency fell consistently on one side of the identity line for the 8 subjects with lung cancer (Figure IV-2B, $p = 0.007$).

Figure IV-1

Comparisons of log G* (rigidity factor) and tan δ (recoil factor), at low frequency, for mucus collected from the mainstem bronchi ipsilateral and contralateral to a defined lesion. Patients with lung cancer are indicated by the filled circles.


Rigidity Factor at 1 rad/s
Ipsi log G*1
Contra log G*1
Non-Ca
Cancer
4
3.5
3
2.5
2
1.5
1
1
1.5
2
2.5
3
3.5
4

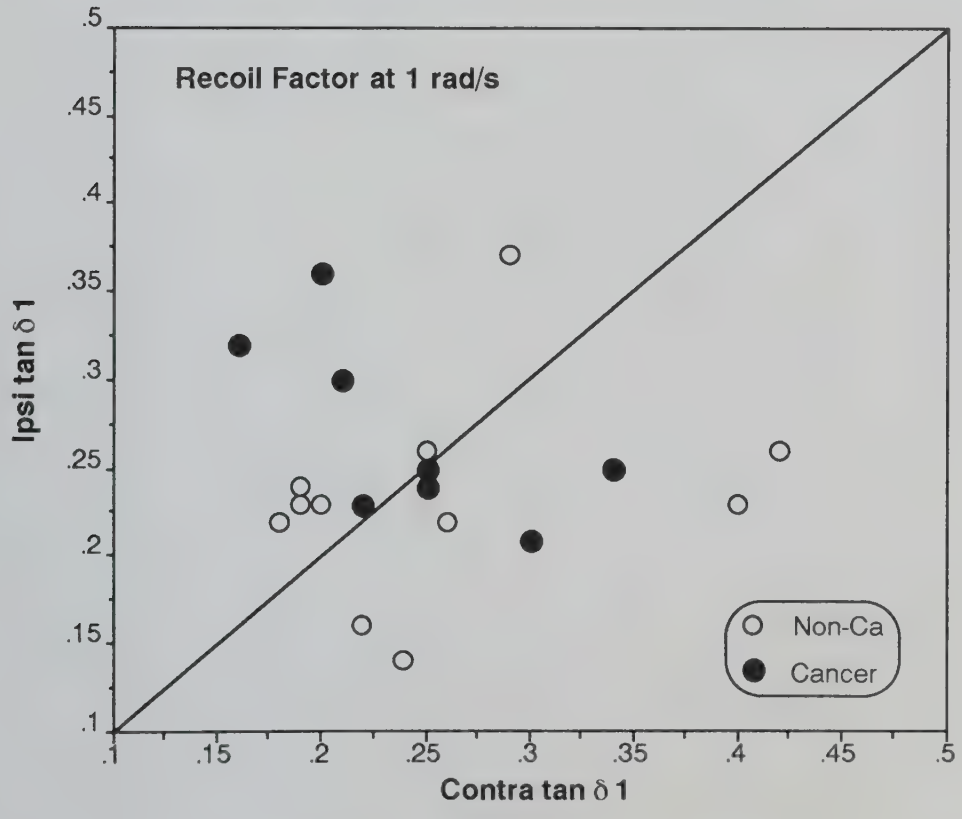
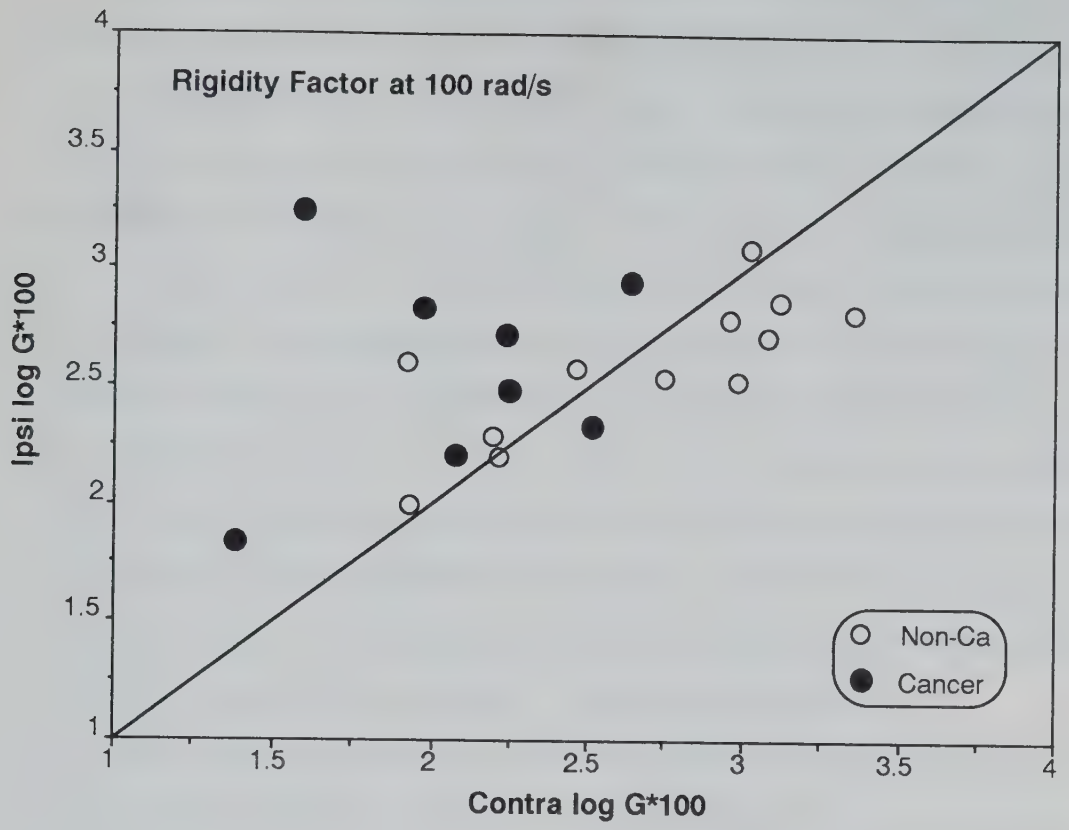
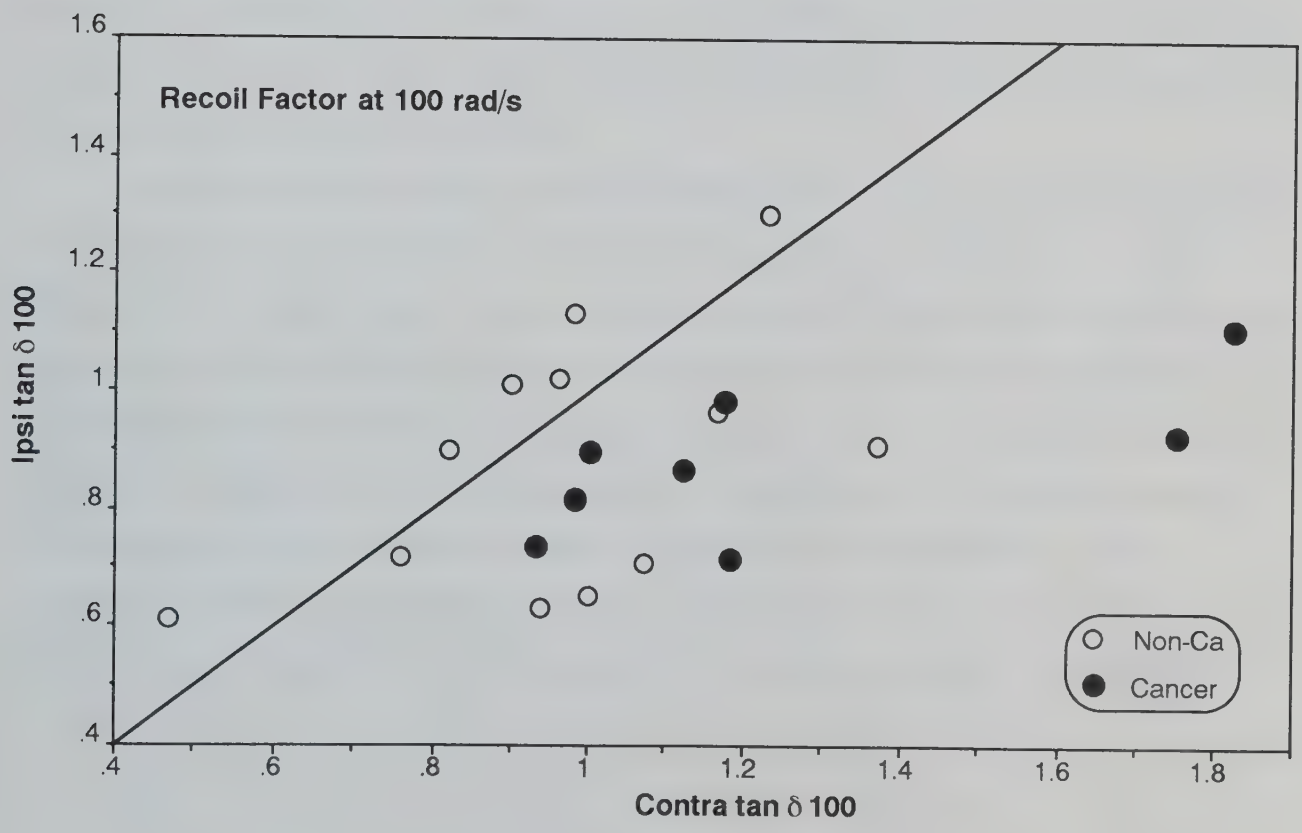

Recoil Factor at 1 rad/s
Ipsi tan δ 1
Contra tan δ 1
Non-Ca
Cancer
.5
.45
.4
.35
.3
.25
.2
.15
.1
.1
.15
.2
.25
.3
.35
.4
.45
.5

Figure IV-2

Comparisons of log G* (rigidity factor) and tan δ (recoil factor), at high frequency, for mucus collected from the mainstem bronchi ipsilateral and contralateral to a defined lesion.


Rigidity Factor at 100 rad/s
Ipsi log G*100
Contra log G*100
Non-Ca
Cancer
4
3.5
3
2.5
2
1.5
1
1
1.5
2
2.5
3
3.5
4


Recoil Factor at 100 rad/s
Ipsi tan δ 100
Contra tan δ 100
Non-Ca
Cancer
1.6
1.4
1.2
1
.8
.6
.4
.4
.6
.8
1
1.2
1.4
1.6
1.8

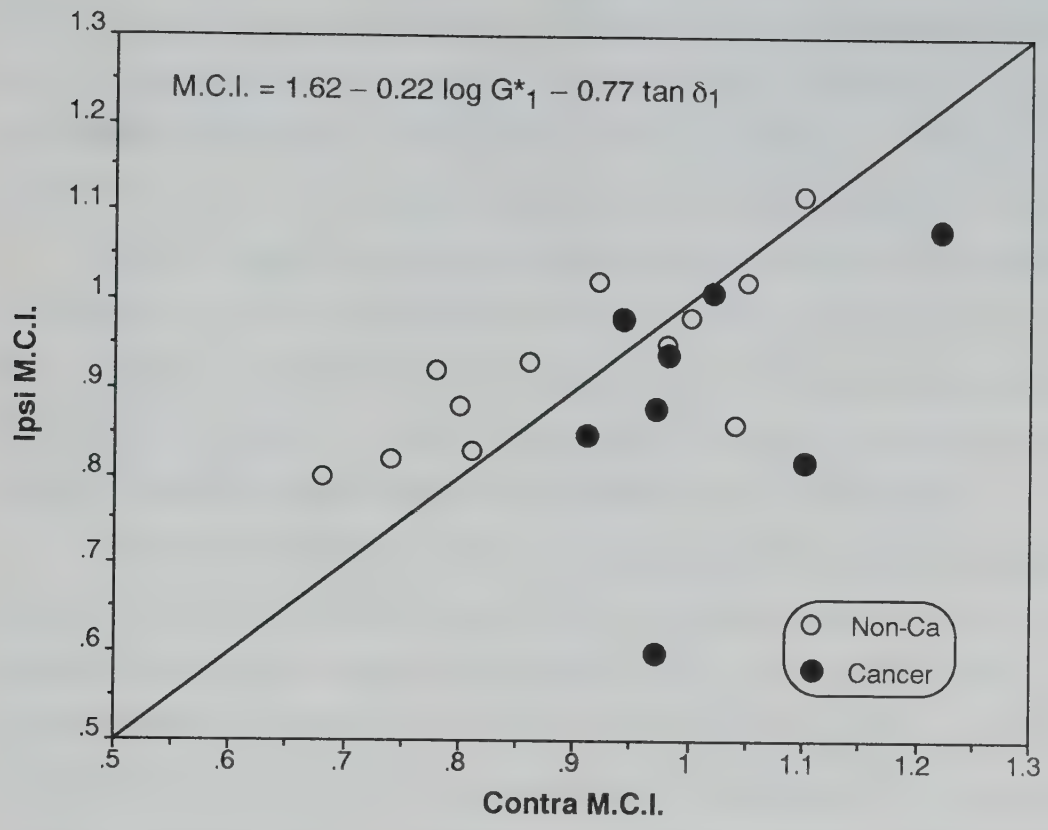
This latter result indicates the mucus in the lung ipsilateral with the cancerous lesion has a lower viscosity/elasticity ratio or greater elastic recoil than the mucus in the contralateral lung. According to equation #2 (see page 66), based on model studies, the mucus from the side with cancer should be more difficult to clear by coughing. The remaining 12 patients (without cancer) of the total of 20 with a side definable lesion did not have mucus that was rheologically distinguishable by site of collection. This was also true of the 18 subjects without a side-definable lesion; in these the inter-site variation in tan δ was generally less than 15 %, similar to the interbronchial variation seen in dogs. [In a comparable sized group of non-exposed dogs, the interbronchial variation in tan δ at 100 rad/s was 18.3 %. In 19 paired mainstem bronchi mucus samples, we observed no statistical differences among all the rheological parameters studied – unpublished data].

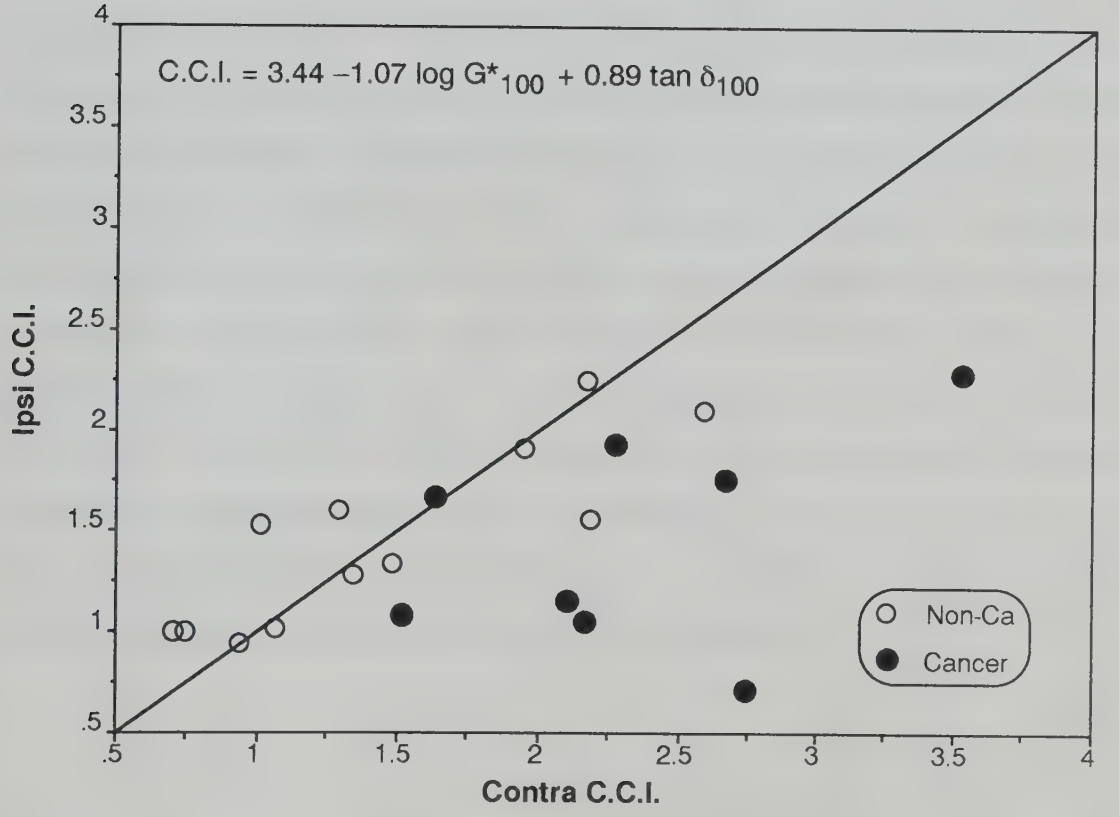
The inter-site variation in the two derived indices of mucus clearability, MCI and CCI, are plotted in Figure 3. Both indices, which are based on combinations of rigidity and recoil, were able to separate the cancer patients from those without, ($p = 0.04$ for MCI and $p = 0.007$ for CCI). In each case, the differences in predicted mucus clearability between the two lungs are such as to disfavor the lung with the cancer.

There was a significantly greater quantity (wet weight) of mucus collected from the side with the lesion in the case of the cancer patients ($p = 0.04$). The percent dry weight in the mucus from the lungs with cancer was marginally less than from the contralateral lungs. Although this was not close to significant, it is interesting that the trend was just the opposite what one would have expected given the differences in rheology between the affected and unaffected sides. The results of the frog palate assay of mucociliary transportability were consistent with the rheological findings, i.e. lowered ciliary clearability on the lesion side, although the differences did not achieve statistical significance.

Figure IV-3

Comparisons of predicted mucociliary clearability (Eq. 1) and cough clearability (Eq. 2) of mucus collected from the mainstem bronchi ipsilateral and contralateral to a defined lesion.


M.C.I. = 1.62 – 0.22 log G*1 – 0.77 tan δ1
Ipsi M.C.I.
Contra M.C.I.
Non-Ca
Cancer
1.3
1.2
1.1
1
.9
.8
.7
.6
.5
.5
.6
.7
.8
.9
1
1.1
1.2
1.3


C.C.I. = 3.44 –1.07 log G*100 + 0.89 tan δ100
Ipsi C.C.I.
Contra C.C.I.
Non-Ca
Cancer
4
3.5
3
2.5
2
1.5
1
.5
.5
1
1.5
2
2.5
3
3.5
4

These latter data are presented in Table IV-1.

There was no consistent variation in rheological properties between the first and second sample taken, nor between the right and left sides (in patients without side-definable lesions). The rheological properties of mucus from the mainstem bronchi were comparable to the properties of tracheal mucus samples obtained from patients with similar smoking histories (5).

A more detailed analysis of the recoil factor at 100 radians/sec is indicated in Figure IV-4. Of the 8 patients with lung cancer, one (G) was a nonsmoker. Two patients (A and H) were only diagnosed with cancer upon followup diagnostic procedures, in one case 3 months after the original bronchoscopy, and the other 20 months later. Details are given in Table IV-2.

The mucus rheological pattern apparently indicative of cancer is actually a triad of rheological criteria: higher tan δ_{100}/lower tan $\delta_{100} > 1.15$, lower value on ipsilateral side, lower value in the range of 0.7 - 1.0. Five of the 13 patients with a side-definable lesion but no cancer had a mucus pattern consistent with this rheological triad, and 4/5 of these could be considered to be at elevated risk to develop lung cancer. In addition, the two patients who either subsequently developed cancer, or in whom cancer was only detected later also fit this rheological risk pattern. The risk level determined was based on the rheological result and on other clinical and non-clinical criteria. The major criteria for risk level, in addition to the mucus rheological pattern, are: cigarette smoke exposure, radiological lesion, anatomopathological changes of the airway cells, exposure to well established carcinogenic factors such as uranium. There exist others that may be considered minor criteria, such as familiar history of lung cancer. Details of these patients are indicated in Table IV-3. Six of the remaining 7 patients who did not exhibit these mucus rheological characteristics were characterized as being at lower risk to develop lung cancer, although given the fact they underwent an indicated bronchoscopy, the assignment of risk must be considered relative.

Table IV-1

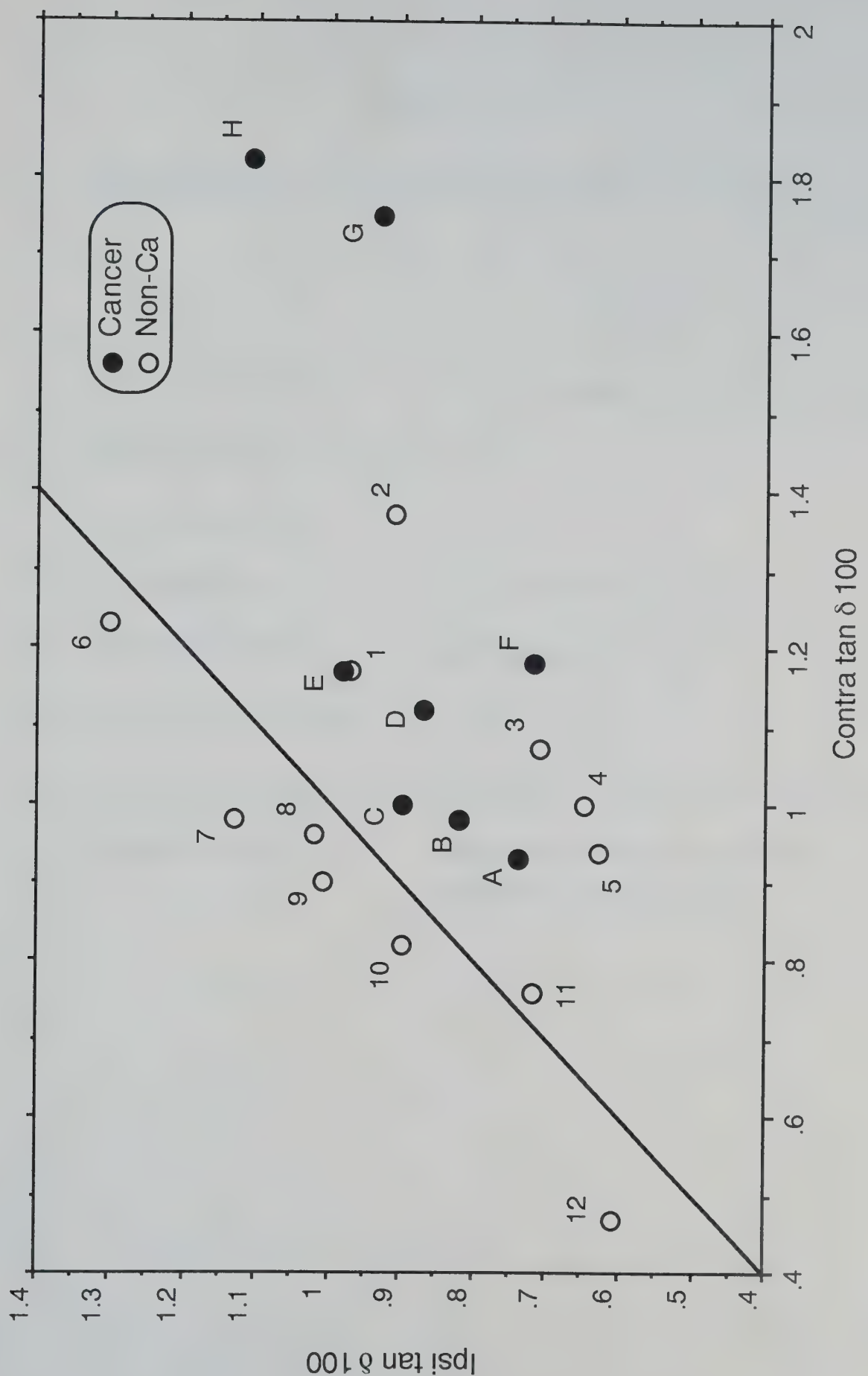
Mucus Volume, Percent Solids Content and Frog Palate Clearability

	Weight of mucus collected (mg)	Solids content (%)	NFPTR
Patients with cancer			
ipsilateral bronchus	32.7±25.3 (6)	10.5±3.6 (6)	0.62±.43 (6)
contralateral bronchus	11.0±7.2 (5)	11.9±6.5 (5)	0.92±.48 (6)
p value	.035	.48	.28
Patients without cancer			
ipsilateral bronchus	6.4±6.5 (12)	11.7±5.0 (9)	0.98±.39 (12)
contralateral bronchus	8.2±8.0 (12)	14.3±5.1 (9)	0.91±.50 (12)
p value	.48	.18	.32

* NFPTR = normalized frog palate transport rate. Data are means±SD, with numbers of observations in brackets. Inter-site comparisons are based on paired *t* tests.

Figure IV-4

Comparison of high frequency recoil factor (tan δ at 100 rad/s) for mucus collected from the mainstem bronchi ipsilateral and contralateral to a defined lesion. The deviation from the line of identity for the cancer patients is significant ($p < 0.007$). The letters beside the filled circles indicate individual cancer patients, as described in Table IV-2. The numbers beside the open circles refer to individual patients without cancer, as described in Table IV-3.


Cancer
Non-Ca
Ipsi tan δ 100
Contra tan δ 100
1.4
1.3
1.2
1.1
1
.9
.8
.7
.6
.5
.4
.4
.6
.8
1
1.2
1.4
1.6
1.8
2
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
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9
10
11
12

Table IV-2

Characteristics of Lung Cancer Patients in Study

	Ca type	Comment
A	RUL Adeno Ca II/III	*Diagnosis made 3 months later*
B	LUL Oat Cell Ca	
C	RLL Small Cell Ca	
D	RML Adeno Ca	'81 RU lobectomy (Adeno)
E	LBr Squam Cell Ca	Radiotherapy for Ca '86
F	Rt Br Typical Carcinoid	
G	LUL Large Cell Anapl Ca	*Nonsmoker*, widespread metastasis
H	Rt Squam Cell Ca	*Diagnosis made 20 months later*, metastasis to both lungs

Table IV-3
Characteristics of Non-Cancer Patients in Study

	Smoking	Rx/Path	Mucus Ca Pattern	Risk for Ca	Comment
1	Non Sm	RUL Infiltrate	√√	High	Benign Tumor Rt L, Resect '55
2	Ex Sm	RLL Nodule RLL Hyperplasia	√√	High	Uranium worker, Hamartoma diagnosed later
3	Ex Sm	R Hyperplasia	√√	High	
4	Non Sm	Benign Atypia	√	Low	
5	Sm	L Squam Atypia	√	High	Pt to be followed cytologically
A	Sm	Dysplast Atypia	√	High	Bladder Ca
H	Sm	RUL Mass	√	High	Rheum Arthritis 2 Brothers w/L Ca
6	Sm	LUL Collapse Squam Metaplasia	x	Low	Bronchitis
7	Non Sm	RLL Mass	x	Low	50 % Obstr, Benign Tumor
8	Non Sm	L Hyperplasia	x	Low	
9	Ex Sm	RLL Nodule	x	High	RLL Hyperplasia
10	Non Sm	Rt Hyperplasia	x	Low	Bronchitis
11	Sm		x	Low	CxR artifact
12	Sm	Rt Hyperplasia	x	Low	Pulm Fibrosis

* Two patients (A and H) originally classified as "high risk" were later diagnosed with lung cancer, and transferred to Table IV-2.

DISCUSSION

In 1957 Hilding (6) presented a work that suggested that alterations in mucociliary clearance might allow carcinogens to be retained in the lung longer than usual, thereby prolonging their action. He also mapped the carcinoma distribution according to this hypothesis, a distribution that matched reasonably well with the more frequent sites of neoplastic transformation. A decade later, Auerbach and coworkers (13) presented evidence for the anatomical lesions that occur to the airway epithelium as a result of cigarette smoke exposure in dogs. These secondary changes occur at both the cellular and tissue levels. In humans these changes take place in a progressive fashion according to the level of cigarette exposure, and over a very prolonged period of time, approximately 30 to 40 years.

The prolonged process of development of lung cancer prevents or makes it almost impossible to undertake a longitudinal or prospective study to prove or disprove either of the two major hypotheses, namely: 1) changes in mucus clearance due to abnormal mucus rheology enhance the possibility of developing lung cancer, or 2) the tissue that has become cancerous then produces abnormal mucus. Although it has its limitations, we consider that the cross-sectional approach may help to decipher this complicated and long process.

In humans, it has not been proven that smoking and the development of lung cancer have a direct causality, although a very close relationship has been established. Smoking-animal models have failed to develop lung cancer; these models, however, have presented the possibility of studying and following the progress of the anatomical changes that predate the neoplastic process.

King *et al* (4) reported changes in the biochemical composition of the mucus of dogs exposed to cigarette smoke, particularly in the galactose content. The galactose was used as a marker of the mucus glycoprotein content. The fall in galactose content occurred at a very early stage and occurred simultaneously with a similar fall in mucus rigidity. Some observers may interpret these changes as a result of the dilution process that happens when the amount of inflammatory fluid increases due to the irritation caused by the

cigarette smoke. After continued smoke exposure, the rigidity of the mucus returned to normal values while the galactose content remained at the same lower level. The prolonged decrease in galactose content in dogs exposed for a longer period of time was interpreted as a change in the nature of the mucus.

Our previous work in patients (5), showed an almost identical pattern regarding a fall in mucus rigidity with an eventual return to normal values. It is tempting to assume that a similar biochemical change might have happened in these subjects as well. It is obvious that this hypothesis for biochemical change must be proven or discarded.

Regarding lung cancer development, we considered some clinical facts: usually a neoplastic process occurs unilaterally and very rarely it is bilateral. It is preceded very often by a unilateral process: pneumonia or pneumothorax. From this information, we thought that we may observe unilateral alterations in mucus rheology. We decided to collect mucus samples from each mainstem bronchus, and perform a comparative analysis.

Data in this study does not disagree with the results obtained in our previous study. We have reaffirmed the alterations observed in both ciliary clearance and cough clearance as a consequence of prolonged smoking. The observed rheological alterations did not permit us to unequivocally identify patients with lung cancer. When used in combination with the other criteria that we considered major, however, we were able to closely match the pathological reports. Even more striking, two patients were confirmed to have lung cancer only after a second biopsy obtained by bronchoscopy, while earlier we considered these patients as having mucus rheological properties consistent with lung cancer, based on our combination of criteria.

It is interesting to note that the recoil factor and rigidity factor equally distinguish the cancerous lung from the lung without detectable cancer, but only the recoil factor (tan δ 100) appears to be capable of distinguishing high risk from low risk patients.

Some researchers have found increased alterations in the mucociliary rate in patients with lung carcinoma when compared with patients without lung cancer matched for cigarette consumption and pulmonary function. They also

reported that mucociliary clearance is even lower on the tumor side. The finding in the present study that mucus on the lesion side was rheologically inappropriate for efficient clearance is thus consistent with the observation of Matthys *et al* (7).

Abnormalities in both of the clearability parameters – ciliary and cough – were seen in most patients with lung cancer. These findings can be considered as evidence to support the hypothesis that prolonged retention of carcinogens due to inadequate clearance of mucus could lead more easily to the development of cancer. Neither parameter was distinctive for lung cancer, since there was a degree of overlap with the population that did not have cancer. It is tempting to speculate, however, that the patients who have mucus inappropriate for clearance will be future targets for the development of cancer if they are exposed unnecessarily to carcinogens.

Our results unfortunately do not allow us to determine whether the development of inappropriate mucus that appears to distinguish patients with lung cancer from others occurred prior to or subsequent to the initiation of the cancer. If the inappropriate mucus predated the cancer, the findings would be strong evidence for the initial hypothesis, i.e. that prolonged retention of carcinogens due to abnormalities in mucus clearance could be a contributing factor to carcinogenesis. On the other hand, if the inappropriate mucus developed concurrently with or subsequently to the initiation of the cancer, the finding of a rheological abnormality would at best merely represent an early, and non-specific, indicator of cancer. Only further studies on a much larger scale will enable us to distinguish between these two possibilities.

Although it was not statistically different, it is interesting to observe the non-concordance between percent solids/wet weight data, and the rheological data, in our group of patients. The larger volume of mucus collected from the ipsilateral side may be explained by the presence of the lesion on that side, resulting in an increased mucus production. Along with this, a lower percentage of solids content, suggesting increased fluid transudation, is not unreasonable. However, according to the rheological data obtained for the ipsilateral side (increased mucus rigidity and recoil), we should have expected

a sharp increase in solids content and not the modest decrease that was observed. We can only explain this non-concordance between percent solids content and rheological results as indicating a mucus "supermacromolecule", or more likely, the production of macromolecular components, protein backbone or oligosaccharides or both, of an entirely different tridimensional orientation, which could give rise to the observed mucus rheological properties. Basbaum and Jany (14) pointed out that airway irritants such as tobacco smoke may modulate, via genetic regulation, the biosynthesis of airway secretions and the induction of enzymes. In biochemical terms, this may involve the upregulation of mucin production, as well as altering the stereometric orientation of the different macromolecular components.

In summary, patients with lung cancer appear to exhibit a distinctive mucus pattern, consistent with an impairment in mucus clearability by coughing, in the lung with the lesion. Some non-cancer patients exhibit a similar pattern of mucus rheological abnormality. Most of those doing so are at an elevated risk of developing lung cancer, based on other clinical criteria, while most of those not exhibiting the abnormal mucus pattern appear to be at much lower risk.

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Chapter V

CONCLUSION

Mucus is a unique *materia* that plays an essential role in maintaining a steady and strictly-controlled homeostasis in several systems of the body. Mucus is present in the airways system, the gastrointestinal system and the genitourinary system. Two types of cells producing mucus are the mucous cells and the serous cells. These secretory cells may be single cells like the goblet cell or they may form groups like the glandular cells. In the lungs, other types of cells such as the Clara cell and the type II pneumocyte, produce materials that contribute to the unique physical properties of mucus (1, 2).

Within these mucus-producing cells are the organelles, which are considered to be the physiological unit responsible for building the mucus macromolecules (3). This microsystem is formed by the rough endoplasmic reticulum, the lysosomes and the Golgi complex (RERLGC). The microsystem acts as a functional unit. It is not known how the unit is controlled nor where the control centre is located.

One controversial view is that mucus production is genetically controlled (4). This perspective is based on the striking similarities between the terminal end of the sugar chains in mucus and the terminal end of the sugar chains of the erythrocytes, which determine the ABO specificity of the blood groups. This viewpoint gains support when we observe certain biochemical alterations of the mucus in non-malignant processes such as metaplasia of the gastric mucosa, as discussed in the Introduction.

Mucus is a dynamic vehicle or medium that interacts directly with various types of cells and tissues in the different systems in which it exists. The dynamic interaction of the mucus and cilia seen in the airways effectively demonstrates this bicellular interaction of the cells: mucus-producing cell with either a ciliated or non-ciliated cell. It is important to note that both mucus and cilia are in fact a product of or part of a cell. Their interaction indirectly reflects the intimate relationship between the binary system of the mucus-producing cell and the ciliated cell. If we wish to understand the bicellular interaction in the airways, we should study the mucus and cilia interaction.

We are using this integrated approach to gain more insight into what occurs when the airways are affected by external factors such as the inhalation

of cigarette smoke. Cigarette smoke produces alterations in ciliated and non-ciliated cells. Evidence for this has been presented by several researchers (5, 6, 7, 8, 9). In our investigations we have found that cigarette smoke also produces alterations in the mucus properties of viscosity and elasticity.

In future research we plan to see if there is a correlation between any alteration in the bicellular interaction of mucus and cilia, and anatomical changes in the mucus-producing microsystem, RERLGC. We hope to eventually determine the structural alterations of the mucus macromolecules associated with smoking and carcinogenesis. This is presently beyond the scope of this study.

The mucus macromolecules has a basic, common and complex biochemical structure. Mucin macromolecules consist of oligosaccharide units attached to a proteic core (10, 11, 12). These macromolecules are built by the same type of cells, and within the cells by a similar microsystem of organelles. Although the main function may vary in different organ systems, mucin macromolecules share a similar structure throughout the body.

We may observe that mucus of the different systems could be affected in a similar fashion by a common factor like cigarette smoke. In the analysis of mucus, it is important to study the stereometric or tridimensional modification that may occur in the macromolecular structure as a result of injurious agents (13). How are the mucus properties altered, how does further damage occur and how does this possibly allow or enhance the development of neoplastic alterations?

The complex biochemical composition of mucus allows it to exert its unique physical properties. Viscosity and elasticity are the physical characteristics considered essential for mucus to function (14), although there are probably other equally important characteristics, such as adhesivity and spinnability. The mucus and cilia system represent the most important line of defense for the respiratory system against inhaled, harmful substances. The mucus layer forms an anatomical/physiological unit with the cilia, thereby constituting the first line of defense of the lungs. The unit acts as a mechanical barrier, trapping foreign material and removing it. The mucociliary system also

acts as the main source of airway humidification, as an ion exchange field, and as a source of immunologic defense. The integrity of this unit is vital for its proper function. Acting as a unit, any change that occurs in one component will produce a different response. The response would be temporal or permanent depending on the type of change or the cause of the change.

Two clearance mechanisms exist in the airways – bicellular or mucociliary clearance and cough clearance. Frequency of mechanical response is important for mucus clearance, particularly in the airways. There is an optimum range among the physical properties that give mucus its capacity to behave in a balanced manner even at very different frequencies or rates of displacement. Low frequency or low amplitude is related to ciliary clearance, while high frequency/high amplitude is more closely related to cough clearance. This bimodal response of the mucus to different frequencies is better explained with a simple and practical analogy: the seat belt. The seat belt responds or functions differently depending on the impulse or tension that is applied. If one pulls it slowly (low frequency), it can be extended easily, but if it is pulled suddenly or sharply (high frequency), it holds one tightly to the seat and is impossible to extend.

In the gastrointestinal tract, there must be a balance between the stability of the mucus as a protective layer, and its capacity to permit absorption of nutrient macromolecules. In the cervix, a balance must also exist between the function of mucus as a barrier and as a facilitator of fertilization.

Several internal or external factors may disrupt this optimum balance or range and create a change that may be progressive and definite. How long it will take for these changes to become irreversible or permanent is still an open question. The structural changes that will lead to reversible or irreversible changes in the composition of the mucus are even more debatable. Although much is known about the chemical and physical properties of mucus, there remains much more to be explored about its function and operation.

Lung cancer remains the leading single killer among all forms of cancer, and any effort to prevent its development cannot be overemphasized (15, 16, 17, 18). The intimate relationship between cigarette smoke exposure and lung

cancer has been widely documented, but no direct causal relationship has yet been proven.

Various large scale screening studies have been carried out in an attempt to find a method of early detection of lung cancer, using several combinations of data including clinical, pathological, biochemical and radiological (19, 20, 21, 22). Many simple as well as sophisticated techniques have been developed with the same purpose, and tremendous financial resources have been devoted to the study of lung cancer (23, 24, 25, 26). Nevertheless, very little has been achieved – the mortality rate is still very high, the 5-year survival is very low, the suffering of the patient and the patient's family is heartbreaking. Yet there are very few studies, if any, that have considered whether the mucus or the mucus interaction plays a protagonistic role in this neoplastic process. Our efforts are oriented toward showing that mucus plays a protagonistic role in this process.

In our first study (Chapter III), we observed a long lasting, progressive alteration in the viscoelastic properties of tracheal mucus with smoking. The clearance function of the tracheal mucus had deteriorated over the years of smoking. This alteration in airway clearance, by cilia or by coughing, would allow tobacco smoke carcinogens to remain longer in contact with the airway epithelium, and supports the hypothesis that the alterations in mucus viscoelastic properties may act as a promoting factor in carcinogenesis.

In this first study, we also found that age *per se* does not affect the mucus properties, and that pulmonary restrictive disease in nonsmokers does not alter the viscoelastic properties of the mucus. The mucus properties in the latter patients are indistinguishable from those of younger and healthy nonsmokers, which we considered to be normal references for our studies.

Smokers, even light smokers (< 1-5 cigarettes/day), show an alteration in the physical properties of the mucus that depends on the exposure or dosage. There is a significant fall in the value of one of the rheological parameters – tan δ 100 – which is related to cough clearance. This alteration enables us to differentiate smokers from nonsmokers. Furthermore, we found that the mucus of cancer patients is inappropriate for both bicellular clearance and cough clearance.

Based on these findings, the second experimental study was designed to further investigate the changes that occur in the bronchial mucus properties as a consequence of smoking and to emphasize the importance of studying these mucus alterations in the developmental process of lung cancer. One crucial step would be to determine what is considered normal for bronchial mucus properties. For this purpose nineteen paired samples were collected from the left and right mainstem bronchi of non-exposed dogs, following the method used to collect tracheal mucus. The same microrheometer technique was used to determine the physical properties of the samples. Using the paired two-tailed t test, no differences were found among all of the rheological parameters analyzed, when comparing left versus right bronchial mucus. [See Figure VI-1].

The data obtained from non-exposed dogs and the data obtained from a comparable sized group of patients without a side-definable lung lesion, who underwent diagnostic bronchoscopy for a variety of reasons, resulted in similar conclusions: the rheological properties of mucus in these groups are not altered. Most of the latter subjects were nonsmokers and some were "healthy" smokers or asymptomatic smokers. The viscoelastic properties of mucus in both groups, the non-exposed dogs and the patients without a side-definable lesion, can then be considered the normal baseline for future studies of mainstem bronchus mucus properties. Our ideal normal reference group should be a much larger group of younger and healthier non-exposed subjects. Our two normal control populations, however, are comparable in size and have almost identical rheological values. Therefore they can be considered representative samples.

The rheological values of samples of mucus obtained from both mainstem bronchi of the subjects exposed to cigarette smoke show alterations of some degree according to the level of exposure. This reaffirms our results obtained in tracheal samples in the first study: cigarette smoke alters the rheological properties of mucus in the two levels studied, trachea and mainstem bronchi. A logical extrapolation to these findings may be to assume that cigarette smoke would affect mucus properties at all levels of the airway tree in a similar manner. This should be tested in future studies.

Figure V-1

Normal Rheological Values for Mainstem Bronchi in Humans and Dogs

Patients Non Exposed Only

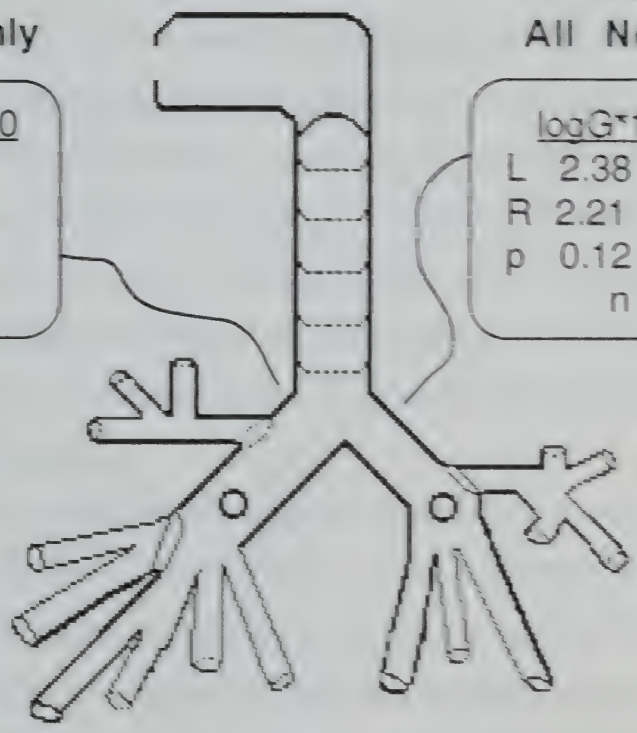
	logG*1	tan δ 100
L	2.31	0.87
R	2.16	0.86
p	0.35	0.77

n = 12

Patients All No Lesion

	logG*1	tan δ 100
L	2.38	0.91
R	2.21	0.88
p	0.12	0.44

n = 18



Non Exposed Dogs

	logG*1	tan δ 100
L	2.34	0.66
R	2.29	0.66
p	0.53	0.94

n = 19

Our initial conclusion from the second study (Chapter IV) is that mucus in the different levels of the airway tree of non-exposed subjects does not have side-dependent rheological or viscoelastic differences for that particular level. There may be interlevel differences, however. We also duplicated our preliminary finding that restrictive airway diseases do not alter the viscoelastic properties of mucus. We reiterate the fact presented in the tracheal analysis that cigarette smoke affects the clearance capacity of the airways proportional to the level of exposure.

The principal aim of the second study was to develop a technique that would enable us to shed light on the multistage complex process in the development of lung cancer. We have considered some clinical and nonclinical aspects that occur during the long developmental process. Lung cancer develops in either lung and very rarely, bilaterally. It is preceded by cytopathological changes, unilateral radiological lesion and very often, unilateral infectious processes. Knowing that tracheal mucus suffers specific alterations in the rheological properties, we assumed that unilateral differences would exist in the physical properties of the mucus on the side with the lesion rather than on the healthy side.

The results confirmed that the changes of mucus in both bronchi were similar to the ones seen at the tracheal level. There were some notable differences, however. We analyzed these differences looking for peculiarities or more specifically for a pattern that could help us to identify people with or without lung cancer. We chose this approach keeping in mind that we are more interested in identifying people who are at *risk* of developing lung cancer. Our reasoning for this interest is that once lung cancer is confirmed there is very little that can be done other than to hope for a maximum five year survival and a lower morbidity rate. If we manage to identify people at risk it may be possible to take measures to slow down the outcome or to prevent the development of lung cancer. This is an ambitious proposition, but we are confident of the potential benefits of our approach towards early detection of malignant processes and other crippling entities.

Using only the rigidity factor and the recoil factor, our approach has

enabled us to distinguish the cancerous lung from the healthy lung. Our results are enhanced when we combine those factors with other criteria that we classify as major contributors such as cigarette smoke exposure, cytopathological changes, unilateral radiological lesions and exposure to other carcinogens like uranium. In two cases where our method suggested that the mucus had identifying characteristics indicative of cancer, the patients were diagnosed as having lung cancer afterwards. One was diagnosed 3 months later, the other 20 months later, after followup tests were carried out.

In carrying out our aim to determine the rheological characteristics of mucus that may identify the risk of developing lung cancer, we further analyzed the rheological parameters. We determined a triad of rheological data, based on the recoil factor at high frequency, that clearly indicated which patients have a pattern of mucus properties indicative of lung cancer. The triad is: higher tan δ 100/lower tan δ 100 >1.15, lower value in the ipsilateral side, and the lower value in the range of 0.7 - 1.0. This rheological triad is closely related to cough clearability and appears to separate low risk and high risk subjects, according to the set of parameters that we have determined. Most of the subjects considered to be at high risk fall on one side of the identity line (Figure IV-4), while the majority at lower risk fall close to the identity line.

The separation is not exclusive of the subjects at risk. One of 7 subjects with the distinctive rheological triad was considered to be at low risk for cancer, while there was one high risk subject among the 7 patients who did not show this rheological pattern. Also one nonsmoker subject with a foreign body in one bronchus showed a similar rheological pattern; his data were excluded from the final analysis because his lesion was acute rather than chronic and it did not relate to smoking and carcinogenesis.

We are confident with the development of our technique. There exist many difficulties yet to be dealt with, but we feel that it has great potential as a valuable tool in the struggle to prevent or fight lung cancer. To summarize, the technique has proven positive in our ability to observe the following:

1) These studies have reaffirmed the important role that unaltered mucus

plays in the homeostatic control of several systems. The physical properties of mucus are critical for the appropriate interaction with other cells and tissues.

2) Cigarette smoke exposure induces measurable changes in tracheobronchial mucus properties which are related to some pathologies. These changes can be detected at different levels of the airway tree. These alterations also enable us to identify subjects exposed to cigarette smoke, even if they have been exposed on a limited dosage.

3) These studies confirm that cigarette smoke exposure affects mucus properties that are basic for clearance of the mucus. These changes occur even at early stages, and give support to the concept that mucociliary clearance is altered as an early step in the cytopathological changes of pulmonary neoplastic processes.

4) Mucus analysis may be valuable as a diagnostic tool in lung cancer. The rheological changes alone may indicate which lung shows pathological alterations suggestive of lung cancer. By combining these changes with other clinical and nonclinical facts, we have enhanced the possibility of a more accurate diagnosis.

5) The most encouraging fact is that our technique enables us to identify people at risk of developing lung cancer. We have reaffirmed the diagnostic and predictive value of the information obtained from the analysis of the respiratory tract mucus. If applied on a large scale, it might encourage those at risk to limit their exposure to carcinogenics, i.e. to quit smoking.

We have also observed several limitations with our technique, including:

1) Bronchoscopic collection of mucus is an invasive technique that is not suitable for repetitive controls. It also limits the studies only to centres that have bronchoscopic facilities. People living in small and poor communities or in developing countries would have restricted access to the benefits of our technique, i.e. the detection of those at risk of developing lung cancer.

2) People who are scheduled to have a bronchoscopic procedure are generally middle-aged people with bronchopulmonary pathology. Younger and healthier people are not scheduled to undergo diagnostic bronchoscopy.

3) There are several technical difficulties or limitations. The small size of the mucus sample makes it vulnerable to deterioration. The brush may irritate the epithelium, which could bleed and hence interfere with the analysis. Irritation can cause coughing, thereby exposing the mucus to a thixotropic effect, which could affect its measured properties.

4) Mucus is a nonhomogeneous substance which may show interaliquot variations. Mucus infections may also cause some variations, which are not yet clearly understood.

5) The cross-sectional approach we have used has many limitations and may not be the best choice for this type of study. A prospective study would be preferable, but the time it takes to develop lung cancer is too long to make its use practical. To find the most suitable method of study is not an easy task. A combination of methods seems to be the most practical approach.

6) The size of our population is another limiting factor. Further studies using a larger population base are needed.

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APPENDIX A
TECHNIQUES FOR MUCOLOGY

Viscoelastic properties of mucus

Definitions: Mucus can be considered a viscoelastic fluid, since it exhibits both liquid-like (viscous) and solid-like (elastic) properties. *Viscosity* is the resistance to flow and represents the capacity of a material to absorb energy as it moves. *Elasticity* is the capacity of a material to store the energy used to move or deform it. The relative proportions of elasticity and viscosity are just as important in describing how a material such as mucus behaves when it is subjected to external forces as are the absolute values of either parameter by itself. *Tangent* δ is the ratio of viscosity to elasticity; this is also known as the loss tangent and can be considered as a recoil factor. *G** is the vector sum of viscosity and elasticity, known as mechanical impedance, which can be termed the rigidity factor.

Cough vs. ciliary clearance: Cilia beat at a frequency of 10-20 Hz and an amplitude of 5 μm while the velocity (frequency times amplitude) in coughing is ~100 times greater. A high tan δ at high frequency suggests a viscous mucus which favors cough clearance. A low tan δ at low frequency characterizes an elastic mucus which favours ciliary clearance. A high G* inhibits both forms of clearance.

Techniques:

The *magnetic microrheometer* is used to measure the viscosity and elasticity of microliter quantities of mucus. A microscopic steel ball is carefully positioned in a 1-10 μL sample of mucus and the motion of this sphere under the influence of an electromagnet is used to calculate the rheologic properties of the mucus. These rheological properties can be used to predict the effectiveness of mucus in clearance.

The *filancemeter* measures large amplitude elastic deformation as thread formation. A high filance (spinnability) value is inversely correlated with cough clearability.

Using a *simulated cough machine*, we are able to measure the recoil effect of a mucus sample by videoscopic analysis and relate this behavior to other measures of mucus viscoelasticity.

Mucus samples are also weighed to determine an index of secretion rate (mucus collection rate, mg/min). Knowing the mucus linear velocity, airway circumference, and collection rate, we can estimate the *in vivo* mucus depth. We use a drying apparatus and microbalance to calculate the % solids content for all samples of mucus larger than 2 μL.

Sample collection

We currently use three methods of collecting mucus samples for analysis:

1) Sputum analysis has been most extensively studied in patients with chronic bronchitis or cystic fibrosis. Although this is a non-invasive technique, drawbacks include frequent bacterial contamination and the inevitable contamination of samples with oral secretions, although this can be minimized by the use of dental cotton. This technique cannot be employed in experimental animals or in humans who cannot expectorate sputum.

2) Bronchoscopy brush collection involves the placing of a standard cytology brush against the airway and removing the brush once it is covered with sufficient mucus for analysis. This technique requires that the animal or patient undergo an invasive procedure, i.e. bronchoscopy. It is also possible that the physical presence of the brush can alter the composition of airway secretions.

3) Endotracheal tube (ETT) collection involves the removal of the mucus coating a freshly removed endotracheal tube. The tubes are usually obtained from patients who require intubation as part of a general anaesthetic. Although this technique does not require that any additional procedure be performed, and sufficient mucus for analysis can be collected even from the youngest patients, the mucus sample may be altered by medications, dry anesthetic gases, and the physical presence of the endotracheal tube, similar to the problem with the bronchoscopy brush.

Ion content (Na, K, Ca, Cl)

The water content of mucus appears to be closely linked to the active transport of ions across the epithelium. Alterations in relative and absolute concentrations of the above ions provide evidence for these processes. Na^+, K^+, and Ca^{++} contents are determined by flame photometry, Cl^- content by

means of a chloride meter. The minimum sample requirement for Na^+ and Cl^- is 10 μL.

Chemical analysis

A detailed chemical analysis is impossible with microsamples of mucus. We can determine, however, the total protein content (Lowry assay) and the galactose content, as an index of glycoprotein concentration, with the microliter quantities of mucus normally available. DNA analysis, which is important in infected samples, is also possible.

***In vitro* ciliary transportability**

We make use of the *in vitro* frog palate assay for both mucus and ciliary function. Using a standard mucus preparation or collected frog mucus we can measure the effect of drugs delivered to the mucosal or air side of the frog palate on the ciliary frequency and transport properties. Alternatively different samples of mucus can be placed on a freshly mucus-depleted frog palate and the transport rate of these samples can be normalized to the transport rate for frog mucus, giving us an assay for the transportability of the mucus sample. Anomalies between frog palate clearability and *in vivo* clearance can give us clues about the interaction between mucus and cilia in experimental situations.

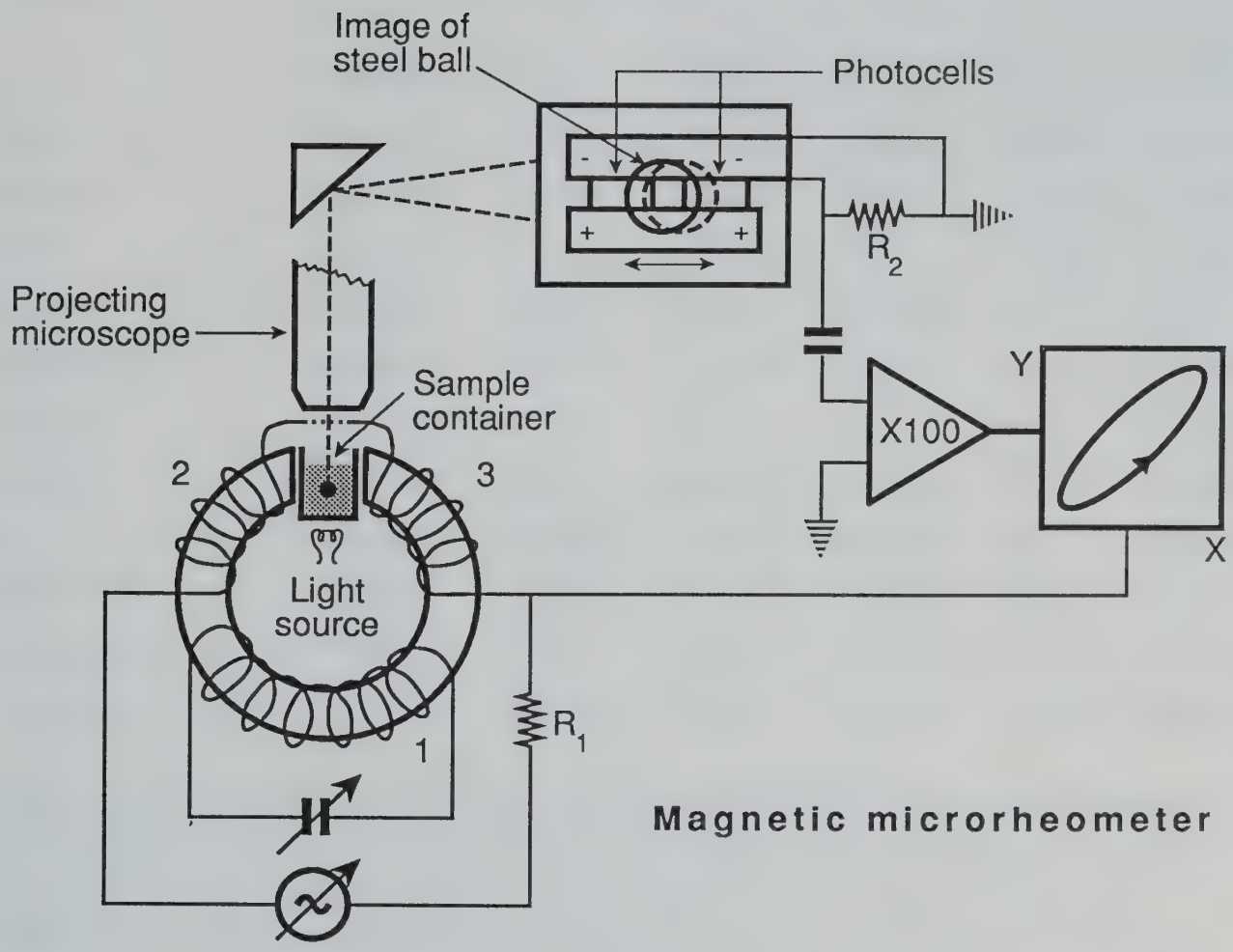
***In vitro* cough transportability**

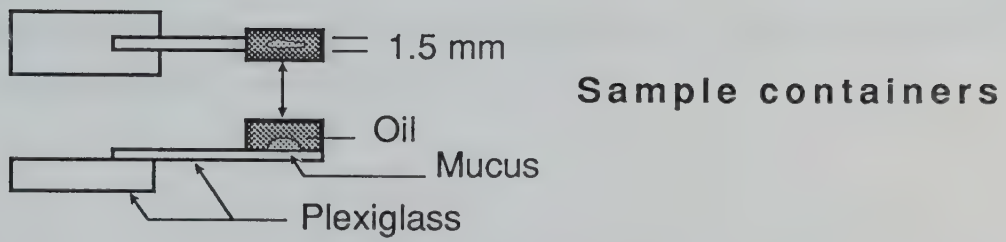
Using a *simulated cough machine* we are able to measure the airflow-dependent clearability and recoil effect of a mucus sample by videoscopic analysis and relate this behavior to other measures of mucus viscoelasticity. Sample requirement is larger than for other forms of analysis, although we have recently reduced this to as little as 30 μL.

Mucociliary clearance rate

In experimental animals, tracheal mucus velocity (TMV, mm/min) can be determined by direct observation of marker particle movement. Mainstem bronchial mucus velocities can also be measured. These are useful in monitoring the effects of unilateral interventions.

Appendix B





Schematic representation of the magnetic microrheometer and the sample containers

APPENDIX C

Summary of Rheological and Transport Parameters (mean ± SD)

	N	log G*,1	tan δ,1	M.C.I.	log G*,100	tan δ,100	C.C.I.
NS(Mtl)	17	2.19±.32	.28±.05	.91±.08	2.58±.33	.95±.26	1.52±.51
NS(Edm)	9	2.12±.40	.27±.03	.94±.09	2.55±.33	.91±.29	1.52±.55
LS(Mtl)	4	1.96±.18	.25±.02	.99±.05	2.30±.18	.79±.08	1.68±.22
LS(Edm)	4	1.85±.25	.23±.04*	1.03±.07*	2.18±.24*	.88±.17	1.89±.34
LS(Mtl+Edm)	8	1.90±.21*	.24±.03*	1.01±.06**	2.24±.21*	.84±.13	1.79±.29
HS(w/oCa)	6	2.27±.18	.23±.04*	.94±.05	2.56±.12	.67±.14*	1.30±.23
XS(w/oCa)	11	1.84±.27**	.24±.04*	1.03±.05***	2.16±.24	.75±.21*	1.78±.38
Ca	12	2.07±.32	.28±.06†	.93±.10	2.37±.31	.72±.16*	1.55±.36
Ca+carcinoid	13	2.05±.31	.29±.06††	.95±.10	2.33±.33	.70±.17**	1.57±.35
HS+XS(w/oCa)	17	1.99±.32	.23±.04	1.00±.07	2.30±.28	.72±.19	1.62±.40
HS+XS(Obstr)	8	1.93±.31	.25±.04	1.00±.07	2.30±.30	.73±.23	1.64±.42

NS(Mtl): healthy nonsmokers (Montreal study, ref. 11); LS(Mtl): healthy young smokers (Montreal study); NS(Edm): nonsmoker patients (present study); LS: light smokers (< 5 cig/day or long-time Ex); HS(w/oCa): heavy smokers without cancer; XS(w/oCa): exsmokers without cancer; Ca: smokers and exsmokers with cancer, including patient with carcinoid; HS+XS(Obstr): smokers and exsmokers without cancer but with low FEV1/FVC. The asterisks indicate significant differences with respect to the normal population: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; the daggers (†) indicate significant differences ($p < 0.05$) with respect to the combined data for smokers and exsmokers without cancer: † $p < 0.05$, †† $p < 0.01$.

	Name	Date	Smoking Hx.	PackYrs	ExYrs	Sex	Age	Ht.	Cancer	Type/OtherDx	FVC	FEV1	FVC(%pred)	FEV1/FVC(%)	logG*1	tan∂1	MCI	lgG*100	tn∂100	CCI
1	E J	86/02/13	Exsm.	30	5	M	46	170	Yes	SmallCellCa Anap	4.00	3.35	88	84	1.69	.20	1.09	1.97	.64	1.90
2	K Z	86/05/14	NonSm.	0	•	M	59	170	No	Sarcoid (PFT July86)	4.20	3.48	98	83	2.75	.32	.77	3.14	.80	.79
3	F M	86/05/15	Sm.	32	•	F	58	160	Yes	AdenoCa, Crohn's, 3 mo. Ex	3.60	2.40	120	67	1.88	.29	.98	2.22	.62	1.62
4	W G	86/05/21	Sm.	50	•	M	64	170	Yes	AdenoCa	4.15	1.95	99	47	2.50	.43	.74	2.92	.56	.81
5	C K	86/06/05	Exsm.	46	11	M	62	171	No	Cigars (12/d,25yr)	4.60	3.19	107	69	2.45	.23	.90	2.69	.44	.95
6	C C	86/06/17	Exsm.	18	20	M	70	•	No		•	•	•	•	1.86	.30	.98	2.19	.83	1.84
7	A C	86/11/24	Sm.	50	•	M	77	180	Yes	SmallCellCa, Asb.Exp.	2.20	1.35	49	61	1.84	.28	1.00	2.24	.64	1.61
8	L S	86/11/24	NonSm.	0	•	F	67	165	No	BreastCa, Pulm.Fib.	1.14	.80	37	70	1.89	.26	1.00	2.36	.72	1.56
9	M F	86/11/26	Sm.	63	•	F	67	168	No		2.42	1.98	75	82	2.59	.25	.86	2.76	.47	.91
10	F M	86/11/26	Exsm.	25	1	M	42	163	No		3.41	2.91	81	85	1.59	.25	1.08	2.02	.99	2.16
11	F B	86/11/27	NonSm.	0	•	F	79	150	No	Pulm.Fib. (Ignore PFT)	.34	.32	•	94	2.06	.25	.97	2.44	.86	1.59
12	R R	86/11/27	NonSm.	0	•	F	62	161	No		2.94	2.34	99	80	2.51	.22	.90	2.78	.86	1.23
13	E C	86/12/01	Sm.	44	•	M	46	175	No	HxTuberc.	4.75	3.67	98	77	2.23	.21	.97	2.49	.59	1.30
14	M S	86/12/01	Sm.	45	•	M	53	175	Yes	LargeCellCa Anap.	3.76	2.39	80	64	2.33	.15	.99	2.35	.60	1.46
15	H S	86/12/05	Exsm.	25	4	M	74	173	No		3.44	2.40	83	70	1.76	.19	1.09	2.02	.55	1.77
16	E K	86/12/08	Exsm.	26	16	F	65	159	Yes	SmallCellCa Anap.	2.38	1.63	85	68	2.31	.30	.88	2.67	.79	1.29
17	L H	86/12/10	Exsm.	36	8	M	74	160	No	Hemopt.	3.20	2.50	95	78	1.55	.27	1.07	1.88	.67	2.02
18	L W	86/12/12	Sm.	17	•	F	53	160	No		3.15	2.50	108	79	2.26	.20	.97	2.61	.61	1.19
19	R R	87/01/22	NonSm.	0	•	M	52	183	No	Bronchiect.	2.87	1.51	55	53	2.47	.29	.85	2.84	.80	1.11
20	A S	87/01/23	Sm.	15	•	F	60	159	Yes	AdenoCa	2.66	1.48	91	56	1.55	.28	1.06	1.92	.93	2.21
21	E R	87/01/23	Exsm.	15	1	F	66	155	No	Asthma	1.50	.70	58	47	2.00	.18	1.04	2.32	.72	1.60
22	S R	87/01/26	Exsm.	40	19	M	74	165	No	Sarcoid, Diabetes	2.97	1.56	81	53	1.55	.29	1.06	1.93	.80	2.09
23	O B	87/01/26	NonSm.	13	30	M	73	172	No	ExYrs >PackYrs	2.59	1.88	63	73	2.07	.20	1.01	2.35	.63	1.49
24	B N	87/01/27	NonSm.	0	•	M	46	195	No	Sarcoid	2.94	2.13	48	72	1.44	.30	1.07	2.01	1.64	2.75
25	M M	87/01/27	NonSm.	0	•	M	24	160	No		2.61	2.36	59	90	2.05	.25	.98	2.45	.84	1.57
26	M M	87/01/28	Sm.	50	•	M	57	175	No		4.07	3.03	88	74	2.09	.29	.94	2.53	.83	1.47
27	A C	87/01/28	NonSm.	3	•	F	53	•	No	1-2cig/d	•	•	•	•	1.84	.27	1.01	2.24	.96	1.90
28	B D	87/01/28	NonSm.	0	•	F	63	163	No		2.58	2.01	85	78	2.00	.27	.97	2.37	.99	1.79
29	A M	87/01/29	Exsm.	15	1	M	30	175	No	Sarcoid, 9 mo. Ex	6.03	4.50	116	75	1.67	.26	1.05	2.15	1.20	2.21
30	E L	87/01/29	NonSm.	2	•	M	45	177	No	Tuberc., 1-2cig/d	•	•	•	•	1.50	.18	1.15	1.83	.93	2.31
31	F P	87/01/30	Sm.	20	•	M	65	170	Yes	AdenoCa	4.37	3.24	105	74	2.12	.29	.93	2.49	.88	1.56
32	E H	87/08/17	Exsm.	60	4	M	55	172	No		3.12	1.86	69	60	2.12	.21	.99	2.39	.76	1.56
33	L D	87/08/17	Sm.	13	•	M	24	189	No	RenalFailure	4.79	4.23	78	88	2.29	.20	.96	2.54	.83	1.46
34	W B	87/08/17	Sm.	40	•	M	53	170	Yes	Squam.CellCa	4.50	3.10	102	69	2.38	.30	.87	2.64	.89	1.41
35	M B	87/08/19	Exsm.	47	16	M	64	•	No		•	•	•	•	1.89	.19	1.06	2.16	.56	1.63
36	E B	87/08/19	NonSm.	0	•	M	76	•	No		4.80	3.30	115	69	1.89	.28	.99	2.56	.71	1.33
37	D P	87/08/19	Sm.	50	•	F	70	157	Yes	LargeCellCa Anap., 3 mo. Ex	1.22	.83	47	68	1.91	.14	1.09	2.09	.44	1.60
38	I S	87/08/19	Exsm.	21	20	M	76	170	No		2.50	1.60	64	64	1.77	.21	1.07	2.01	.72	1.93
39	K W	87/08/20	Sm.	•	•	F	52	•	No	Stopped 4 mo., no Hx avail.	•	•	•	•	2.13	.22	.98	2.42	.71	1.48
40	A B	87/08/20	Exsm.	40	5	F	61	166	Yes	LungCarcinoid, Crohn's	2.70	2.10	83	78	1.78	.32	.98	1.91	.47	1.81
41	M P	87/09/25	Exsm.	38	1	M	63	178	Yes	OatCellCa, Asbestosis	4.21	2.53	95	60	1.90	.22	1.03	2.22	.90	1.87
42	P P	87/09/30	NonSm.	1	•	F	32	162	No	Asthma, 1 PackYr	2.81	1.94	77	69	1.97	.25	.99	2.29	1.00	1.88
43	G A	87/09/30	Sm.	60	•	M	74	162	Yes	Squam.CellCa	3.15	1.70	90	54	2.44	.23	.91	2.68	.77	1.26

	Name	Date	Imp. Dx.	Source	Lesion	Malignancy	Smoking Hx.	PackYrs	ExYrs	Sex	Age	Comments	log G*1	tanδ1	MCI	logG100	tanδ100	CCI	NFPTR	WetWt.	%Solids	tan100L/S
1	NC	88/05/18	Rt Hil mass/RUL Beng Atyp	Right	Ipsila...	Non CA	Ex Smoker	40	27	Male	61	Trans Cell Ca Bladder	2.06	.16	1.04	2.26	.71	1.65	.82	.82	•	•
2		88/05/18		Left	Contr...	Non CA	Ex Smoker	40	27	Male	61		•	•	•	•	•	•	•	•	•	•
3	FD	88/05/18	Lt Mainst Br Sq C Ca	Left	Ipsila...	CA	Smoker	27	•	Male	74	Radiated (86)	1.98	.21	1.01	2.21	.98	1.95	•	•	•	•
4		88/05/18		Right	Contr...	CA	Smoker	27	•	Male	74		1.63	.30	1.02	2.07	1.17	2.27	•	•	•	1.19
5	MS	88/05/18	Rt Br Typical Carcinoid	Right	Ipsila...	CA	Smoker	18	•	Fe...	84	Typical Carcinoid	2.45	.25	.88	2.73	.72	1.16	•	•	•	•
6		88/05/18		Left	Contr...	CA	Smoker	18	•	Fe...	84		2.04	.25	.97	2.23	1.18	2.10	•	•	•	1.64
7	LF	88/05/19	Chr prod cough/Bilat Atel	Left	•	Non CA	Smoker	24	•	Male	62	Alcoh liver disease	1.75	.27	1.02	2.15	.87	1.91	1.00	2.22	11.26	1.07
8		88/05/19		Right	•	Non CA	Smoker	24	•	Male	62		2.11	.20	.99	2.55	.81	1.43	1.00	2.58	10.47	•
9	WN	88/05/19	Susp AdenoCA	Right	Contr...	Non CA	Non Smo...	3	48	Male	74	Sm x 7mo	•	•	•	•	•	•	.51	•	•	•
10		88/05/19		Left	Ipsila...	Non CA	Non Smo...	3	48	Male	74		2.26	.22	.94	2.50	.91	1.57	.87	2.53	13.83	•
11	GD1	88/05/25	Chr Br Pulm Fib	Right	Ipsila...	Non CA	Smoker	53	•	Fe...	49	Rt Hyperpl Resv Cell	2.54	.22	.88	2.79	.61	1.00	.63	2.85	17.54	1.30
12		88/05/25		Left	Contr...	Non CA	Smoker	53	•	Fe...	49		2.74	.26	.80	2.95	.47	.70	.57	.82	•	•
13	JB	88/05/26	Sarcoidosis	Right	•	Non CA	Smoker	46	•	Fe...	42		2.21	.27	.92	2.50	.62	1.32	1.58	1.80	•	•
14		88/05/26		Left	•	Non CA	Smoker	46	•	Fe...	42		2.57	.33	.79	2.88	.83	1.10	.55	•	•	1.34
15	SA	88/05/26	Non spec cough	Left	•	Non CA	Non Smo...	0	•	Fe...	43		2.18	.21	.97	2.39	.79	1.59	1.50	3.17	19.24	1.20
16		88/05/26		Right	•	Non CA	Non Smo...	0	•	Fe...	43		2.46	.23	.89	2.72	.66	1.12	.69	•	•	•
17	BM	88/06/16	Granuloma tracheost	Left	•	Non CA	Non Smo...	0	•	Male	30		2.13	.27	.93	2.49	1.00	1.67	1.58	9.11	16.14	1.12
18		88/06/16		Right	•	Non CA	Non Smo...	0	•	Male	30		2.72	.26	.81	3.01	.89	1.01	2.03	6.46	24.92	•
19	ET	88/06/17	Unres Rt Pneum/	Left	Contr...	Non CA	Non Smo...	0	•	Fe...	80	CXR Infiltrate RUL/	2.72	.20	.86	2.98	1.17	1.29	.64	1.88	•	1.21
20		88/06/17	- Beng tum res.55	Right	Ipsila...	Non CA	Non Smo...	0	•	Fe...	80	- no change 1991	2.29	.23	.93	2.53	.97	1.60	.66	1.02	•	•
21	WS1	88/06/17	Viral Pneum/ Pers cough	Left	Ipsila...	Non CA	Non Smo...	0	•	Male	51		2.37	.22	.92	2.82	.65	1.00	.59	.33	•	•
22		88/06/17		Right	Contr...	Non CA	Non Smo...	0	•	Male	51		3.13	.18	.78	3.35	1.00	.75	.43	.24	•	1.54
23	CH	88/06/20	Pulm Fibrosis	Left	•	Non CA	Ex Smoker	50	7	Male	69	Chr Prod Cough	2.87	.25	.79	3.09	1.02	1.04	.71	4.19	17.66	1.00
24		88/06/20		Right	•	Non CA	Ex Smoker	50	7	Male	69		2.58	.30	.81	2.96	1.02	1.18	.36	4.87	12.11	•
25	WS2	88/06/22	Asth+Bronchiect	Left	•	Non CA	Ex Smoker	10	26	Male	69		2.02	.52	.78	2.75	1.54	1.87	.47	4.46	8.52	1.20
26		88/06/22		Right	•	Non CA	Ex Smoker	10	26	Male	69		1.74	.35	.96	1.93	1.28	2.51	.55	4.64	14.66	•
27	CG	88/06/23	RUL mass/ Reum Arth	Right	Ipsila...	CA	Smoker	50	•	Male	67	2 Bro w/LCa /	2.13	.25	.95	2.49	1.11	1.76	.74	22.16	9.88	•
28		88/06/23		Left	Contr...	CA	Smoker	50	•	Male	67	- RU lobX 21 mo later/Sq cell Ca	1.68	.34	.98	2.24	1.82	2.66	.67	11.87	6.32	1.64
29	AH	88/06/24	RLL Small Cell Ca	Left	Contr...	CA	Smoker	60	•	Male	58		2.30	.25	.91	2.63	1.00	1.52	.30	•	•	1.11
30		88/06/24		Right	Ipsila...	CA	Smoker	60	•	Male	58		2.61	.24	.85	2.95	.90	1.08	.27	8.73	13.75	•
31	KC	88/06/29	R Pleural infection	Left	•	Non CA	Non Smo...	0	•	Male	65		2.48	.20	.91	2.70	.96	1.41	.67	.52	•	•
32		88/06/29		Right	•	Non CA	Non Smo...	0	•	Male	65		2.39	.32	.84	2.74	1.11	1.50	.55	1.03	•	1.16
33	FH	88/06/29	RLL mass 50% obstr	Left	Contr...	Non CA	Non Smo...	0	•	Fe...	63	Prob Lung Ca/cytology benign	2.63	.29	.81	3.08	.98	1.02	.57	7.26	19.28	•
34		88/06/29		Right	Ipsila...	Non CA	Non Smo...	0	•	Fe...	63		2.27	.37	.83	2.72	1.13	1.54	.67	1.57	•	1.15
35	BH	88/06/30	Sarcoidosis	Left	•	Non CA	Non Smo...	0	•	Fe...	61		2.70	.32	.77	3.01	1.08	1.18	.84	5.32	15.60	•
36		88/06/30		Right	•	Non CA	Non Smo...	0	•	Fe...	61		•	•	•	•	•	•	.39	1.37	•	•
37	WS3	88/07/13	Unres Pneum	Left	•	Non CA	Non Smo...	0	•	Male	70	30 y ago pipe/never inhaled	1.78	.17	1.09	2.34	1.13	1.94	.54	8.89	10.69	•
38		88/07/13		Right	•	Non CA	Non Smo...	0	•	Male	70		1.67	.13	1.14	2.03	1.35	2.47	.30	7.37	12.62	1.20
39	EL	88/07/13	Trach Stenosis	Left	Contr...	Non CA	Ex Smoker	18	1	Male	51	Rt Basal Hyperplasia/	2.79	.42	.68	3.11	1.07	1.06	.17	3.28	24.70	1.51
40		88/07/13		Right	Ipsila...	Non CA	Ex Smoker	18	1	Male	51	- no change-sleep apnea	2.77	.26	.80	2.86	.71	1.01	.71	4.82	19.09	•
41	PC1	88/07/13	RUL Adeno Ca II/III	Right	Ipsila...	CA	Smoker	60	•	Male	64	Papillary C Ca Bladder	2.47	.32	.82	2.84	.74	1.06	.27	81.76	9.65	•

WetWt.	%Solids	tan100L/S
23.16	5.92	1.26
29.95	10.55	1.05
8.28	12.92	•
5.96	14.43	1.29
31.93	12.97	•
23.53	12.87	•
5.51	21.60	1.20
2.60	25.00	1.31
17.09	20.13	•
•	•	•
.93	•	1.09
7.91	16.81	•
8.16	4.78	1.06
4.29	13.29	1.49
2.16	5.56	•
19.32	10.46	1.05
8.04	12.69	•
10.53	10.64	1.51
5.63	9.77	•
11.64	12.46	•
3.80	15.26	1.12
19.24	9.46	1.10
11.96	8.44	•
3.93	14.25	1.00
2.06	27.18	•
27.86	4.02	•
8.68	11.41	1.88
2.87	14.29	1.34
1.97	•	•
2.86	19.58	1.04
1.14	•	•
1.37	•	•
.77	•	1.18
•	•	•
6.46	23.21	1.36
11.44	13.46	1.14
13.96	9.03	•
2.52	16.67	•
14.18	9.24	1.11
29.08	14.58	•
31.56	11.66	1.27

	Name	Date	Imp. Dx.	Source	Lesion	Malignancy	Smoking Hx.	PackYrs	ExYrs	Sex	Age	Comments	log G*1	tan∂1	MCI	logG100	tan∂100	CCI	NFPTR	WetWt.	%Solids	tan100L/S
42		88/07/13		Left	Contr...	CA	Smoker	60	•	Male	64	- Dx later	1.76	.16	1.10	1.97	.93	2.16	.71	23.16	5.92	1.26
43	LD	88/07/19	LLL Nod	Right	Contr...	Non CA	Smoker	•	•	Male	48		2.29	.22	.94	2.46	.76	1.48	.59	29.95	10.55	1.05
44		88/07/19		Left	Ipsila...	Non CA	Smoker	•	•	Male	48		2.34	.16	.98	2.57	.72	1.33	.80	8.28	12.92	•
45	ML	88/07/20	Rad post AdenoCa	Left	Contr...	CA	Ex Smoker	15	6	Male	41	RML Dysplasia	2.20	.20	.97	1.59	1.12	2.74	1.68	5.96	14.43	1.29
46		88/07/20		Right	Ipsila...	CA	Ex Smoker	15	6	Male	41		3.35	.36	.60	3.26	.87	.73	.21	31.93	12.97	•
47	LK	88/07/20	Chr Br Oat C Ca LUL	Left	Ipsila...	CA	Smoker	40	•	Fe...	57	8 mo chg resp funct	2.01	.30	.94	2.33	.82	1.68	1.23	23.53	12.87	•
48		88/07/20		Right	Contr...	CA	Smoker	40	•	Fe...	57		2.16	.21	.98	2.51	.98	1.63	1.16	5.51	21.60	1.20
49	LS	88/07/22	Bronchitis	Right	•	Non CA	Smoker	•	•	Male	48	Sm occasional cigars	2.61	.33	.78	2.98	.92	1.07	.18	2.60	25.00	1.31
50		88/07/22		Left	•	Non CA	Smoker	•	•	Male	48		2.95	.35	.70	3.16	.70	.68	.18	17.09	20.13	•
51	LO	88/08/03	Pulm Hypert I	Right	•	Non CA	Ex Smoker	50	8	Male	43	Lt + Rt Resv C Hyperpl	2.58	.23	.86	2.84	.80	1.11	.93	•	•	•
52		88/08/03		Left	•	Non CA	Ex Smoker	50	8	Male	43		3.04	.37	.66	3.53	.87	.44	.53	.93	•	1.09
53	JG	88/08/04	Bronchitis	Right	Contr...	Non CA	Non Smo...	0	•	Male	47	Lt Basal C Hyperpl/	1.93	.25	1.00	2.19	.96	1.95	1.54	7.91	16.81	•
54		88/08/04		Left	Ipsila...	Non CA	Non Smo...	0	•	Male	47	- no change 1991	1.94	.26	.98	2.28	1.02	1.91	1.35	8.16	4.78	1.06
55	PC2	88/08/04	Chr Br Lt Squam Atypia	Right	Contr...	Non CA	Smoker	35	•	Male	45	To be followed Cyt	2.48	.19	.92	2.74	.94	1.34	1.63	4.29	13.29	1.49
56		88/08/04		Left	Ipsila...	Non CA	Smoker	35	•	Male	45		1.85	.24	1.02	2.54	.63	1.28	1.84	2.16	5.56	•
57	BT	88/08/16	Bronchitis/LUL Collapse	Left	Ipsila...	Non CA	Smoker	50	•	Fe...	61	Sp Cyt/Squam Metapl	1.91	.23	1.02	2.20	1.30	2.24	1.42	19.32	10.46	1.05
58		88/08/16		Right	Contr...	Non CA	Smoker	50	•	Fe...	61		1.89	.19	1.05	2.21	1.23	2.17	.93	8.04	12.69	•
59	WB	88/08/16	Uranium work/RLL Nod	Left	Contr...	Non CA	Ex Smoker	20	12	Male	64	RLL Resv C Hyperpl/	1.47	.24	1.10	1.93	1.37	2.59	1.15	10.53	10.64	1.51
60		88/08/16		Right	Ipsila...	Non CA	Ex Smoker	20	12	Male	64	- RL lobX/beng hamartoma	1.74	.14	1.12	2.00	.91	2.11	.94	5.63	9.77	•
61	MA	88/08/17	RLL Nod	Left	Contr...	Non CA	Ex Smoker	15	3	Fe...	65	RLL Resv C Hyperpl	1.59	.29	1.04	1.92	.90	2.19	1.63	11.64	12.46	•
62		88/08/17		Right	Ipsila...	Non CA	Ex Smoker	15	3	Fe...	65		2.12	.37	.86	2.60	1.01	1.56	1.14	3.80	15.26	1.12
63	GD2	88/08/18	Bronchitis/Cough	Right	Ipsila...	Non CA	Non Smo...	0	•	Fe...	62	Rt Br Basal C Hyperpl/	2.77	.23	.82	3.09	.90	.93	.96	19.24	9.46	1.10
64		88/08/18		Left	Contr...	Non CA	Non Smo...	0	•	Fe...	62	- no change 1991	2.58	.40	.74	3.02	.82	.94	1.11	11.96	8.44	•
65	RL	88/08/22	Hemoptysis Bronchiect	Left	•	Non CA	Non Smo...	0	•	Male	85		1.90	.30	.97	2.30	1.14	1.99	1.24	3.93	14.25	1.00
66		88/08/22		Right	•	Non CA	Non Smo...	0	•	Male	85		2.61	.40	.73	2.30	1.14	1.99	.71	2.06	27.18	•
67	WH	88/08/23	LUL Lg Cell Anapl Ca	Left	Ipsila...	CA	Non Smo...	0	•	Male	65	Wide spread metast	1.61	.23	1.08	1.84	.93	2.30	.97	27.86	4.02	•
68		88/08/23		Right	Contr...	CA	Non Smo...	0	•	Male	65		1.01	.22	1.22	1.38	1.75	3.52	.97	8.68	11.41	1.88
69	CY	88/08/23	Hemoptysis	Left	•	Non CA	Non Smo...	0	•	Male	64		1.93	.25	.99	2.36	1.21	1.99	1.21	2.87	14.29	1.34
70		88/08/23		Right	•	Non CA	Non Smo...	0	•	Male	64		1.97	.22	1.01	2.30	.90	1.78	1.00	1.97	•	•
71	MR	88/08/24	Hx TB Rheum Arth	Left	•	Non CA	Non Smo...	0	•	Fe...	81	Rt Br Basal Hyperpl	2.25	.21	.95	2.55	.79	1.41	.89	2.86	19.58	1.04
72		88/08/24		Right	•	Non CA	Non Smo...	0	•	Fe...	81		1.43	.16	1.17	1.71	.76	2.29	1.02	1.14	•	•
73	PC	88/08/25	Interst Fib Rheum Diab	Left	•	Non CA	Non Smo...	0	•	Fe...	66		3.57	.18	.69	3.85	.51	-.23	.80	1.37	•	•
74		88/08/25		Right	•	Non CA	Non Smo...	0	•	Fe...	66		2.91	.23	.79	3.15	.67	.67	1.35	.77	•	1.18
75	AB1	88/08/30	Breast Ca Cough	Right	•	Non CA	Non Smo...	0	•	Fe...	55		1.88	.22	1.03	2.17	.66	1.71	•	•	•	•
76		88/08/30		Left	•	Non CA	Non Smo...	0	•	Fe...	55		2.63	.38	.74	3.00	.90	1.03	.89	6.46	23.21	1.36
77	WD	88/09/01	Card Arrhythmia	Left	•	Non CA	Non Smo...	0	•	Male	67		1.78	.19	1.07	2.10	.87	1.97	2.53	11.44	13.46	1.14
78		88/09/01		Right	•	Non CA	Non Smo...	0	•	Male	67		1.62	.18	1.11	1.99	.76	1.99	.87	13.96	9.03	•
79	EM	88/09/01	Aortic Stenosis	Left	•	Non CA	Non Smo...	5	40	Male	62		2.81	.13	.89	2.93	.63	.87	.84	2.52	16.67	•
80		88/09/01		Right	•	Non CA	Non Smo...	5	40	Male	62		2.19	.23	.95	2.51	.70	1.38	2.05	14.18	9.24	1.11
81	AB2	88/09/01	Sarcoidosis	Left	•	Non CA	Non Smo...	0	•	Male	55		2.24	.21	.95	2.58	.56	1.18	1.29	29.08	14.58	•
82		88/09/01		Right	•	Non CA	Non Smo...	0	•	Male	55		2.11	.33	.90	2.56	.71	1.33	1.39	31.56	11.66	1.27

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